EXPLORING THE RELATIONSHIP BETWEEN DIET AND OSTEOPOROSIS IN MEDIEVAL PORTUGAL USING STABLE ISOTOPE ANALYSIS

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11 August 2015
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A

THESIS

Presented to the Faculty

of the University of Alaska Fairbanks

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF ARTS

By

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Fairbanks, AK

August 2015

Abstract

This project investigates the relationship between health and diet in medieval Portugal by combining data on the occurrence of osteoporosis with information on past diet derived from stable isotope ratios. The aim of this project is to identify whether different sources of protein influenced the prevalence of osteoporosis in three populations. Individuals from three different regions of Portugal were previously evaluated for bone mineral density at the University of Coimbra, Portugal, and bone samples from 91 of these individuals underwent stable isotope analysis at the University of Alaska Fairbanks. Collagen suitable for isotopic analysis was extracted from all individuals and indicated a negative correlation between bone mineral density (BMD) and carbon and nitrogen isotope values for females at one site and a positive correlation for males at another site. These results, combined with the lack of a clear relationship between BMD and nitrogen isotope values for the other subgroups, suggest a complicated relationship between dietary protein source and the occurrence of osteoporosis. While samples sizes are small, the data indicate that future analysis is warranted, particularly considering the high incidence of osteoporosis and the economic and individual strain of the disease.

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Acknowledgments

I would like to express my very great appreciation to my committee Dr. Jamie Clark, Dr. Carrin Halffman, and Dr. Brian Hemphill for their patient guidance, enthusiastic encouragement, and useful critiques of this research work.

I would also like to extend my deep gratitude to Dr. Francisco Curate. Without his willingness to present resources and spend time so generously, this project would never have transpired.

Finally, I wish to thank my friends and classmates, Erin Dinneen, Carla Frare, Crystal Glassburn, and Joe Keeney for their support, encouragement, and random kitten photos throughout my study. Without you, I would have been lost.

Chapter 1 INTRODUCTION

This study investigates the relationship between health and diet in medieval Portugal by combining data on the occurrence of osteoporosis with information on past diet derived from stable isotope ratios. The aim of this study is to identify whether different sources of protein influenced the prevalence of osteoporosis. This information is particularly important because modern clinical studies on the relationship between dietary protein and osteoporosis have presented conflicting results.

To address the question of how protein affects bone density, I analyzed medieval individuals from three different regions of Portugal. A total of 91 individuals underwent stable isotope analysis at the University of Alaska Fairbanks, and 66 individuals were previously evaluated for bone mineral density at the University of Coimbra, Portugal. Of those individuals in which bone mineral density values were available, I completed statistical analysis of stable isotope ratios and density values to check for patterns of association.

This study presents a historical perspective on a disease that affects millions of people today. While cultural and social norms have changed over the centuries, cases of osteoporosis have persisted. Although the relationship between diet and osteoporosis is complicated, this study explores aspects of this relationship that clinical studies are unable to address. In addition to providing information on the prevalence of osteoporosis in past populations, this study also contributes to clinical research on the disease's etiology.

Osteoporosis literally translates from Latin as "porous bone" (Holick & Dawson-Hughes, 2004). It is a systemic metabolic skeletal disease, characterized by a reduction in bone mass and a deterioration of the bone tissue (Agarwal & Glencross, 2010). While osteoporosis is defined as a loss of total mass, for practical purposes it is diagnosed through measures of bone mineral density. Where the density and quality of bone are compromised, bone fragility and propensity to fracture consequently increase, especially in the areas of the wrist, hip, and vertebrae (Brickley & Ives, 2008). As a living tissue, bone is constantly remodeled over the years, and osteoporosis and osteopenia (e.g., reduction of bone density without the propensity of fracture) occurs when the creation of new bone does not keep up with the removal of old bone. This condition is age-related: usually manifested after menopause in women; and in the seventh and or eighth decade in men (Rosen, 2004).

Three skeletal regions are recognized as osteoporosis-related fracture sites: the vertebrae, femoral neck, and distal radius (Lenchik, Vatti, & Register, 2004; Melton, Crowson, O'Fallon,

Wahner, & Riggs, 2003). However, osteoporosis potentially increases susceptibility to fracture anywhere in the skeleton (Cummings & Melton, 2002). Fractures can be diagnosed archaeologically by visual examination of complete bones, but this process can become complicated when attempting to determine peri- versus postmortem fracture. Given this, bone mineral density is a useful measure for identifying osteoporosis and its precursor osteopenia among both modern and archaeological populations. Through the use of densitometric devices, bone mineral density can be analyzed and compared to reference values for healthy adults in order to identify individuals who suffer from the disease.

Osteoporosis is a significant concern in modern societies and is currently the most common bone disease in humans (National Osteoporosis Foundation, 2010). Approximately one out of every two women over the age of 50 will experience an osteoporosis-related fracture at some point in her lifetime, as will approximately one in five men over the age of 70 (U.S. Department of Health & Human Services, 2004).

Not only does osteoporosis affect these individuals and their families, but it also places great strain on the economy. The United States alone spends nearly $14 billion each year for the treatment of osteoporosis complications, and as life expectancy increases there will be a stepwise increase in the financial burden on society (Ray, Chan, Thamer, & Melton, 1997). It is estimated that by the year 2050 the cost will have risen to $131 billion (Johnell, 1997).

Because osteoporosis is associated with significant morbidity, mortality, and expenses, it is vital to investigate factors that may exacerbate the disease. Extensive studies on the relationship between diet and bone mineral density indicate that diet may affect osteoporosis risk by impacting homeostasis and therefore bone turnover rates (Holick & Dawson-Hughes, 2004). While clinical studies of diet and osteoporosis have focused on the intake of micronutrients, such as calcium and Vitamin D, other nutritional factors may be important, including the amount and source of protein. Specifically, protein intake and its source may affect bone in several ways: protein provides the structural matrix of bone, and certain types of protein may optimize the insulin-like growth factor (aka IGF-1, which stimulates bone growth), and have been reported to increase urinary calcium and intestinal calcium absorption (Heaney & Layman, 2008). Obviously important to bone health, the impact of different dietary protein sources on bone mineral density has been a point of controversy for decades, and various sources of protein have been identified as being both detrimental and beneficial to bone health (Barr, 2004; Fernandes, 2004; Larsson, Wolk, Brismar, & Wolk, 2005; New

et al., 2000; Sellmeyer, Stone, Sebastian, & Cummings, 2001; Tucker et al., 1999; Wachman & Bernstein, 1968).

Some clinical studies have suggested that animal protein has a detrimental effect on bone mineral density because it induces an increase in urinary calcium excretion, while others have found that it has a positive effect because it is associated with IGF-1, which may have an osteotrophic effect (Larsson et al., 2005; New et al., 2000; Sellmeyer et al., 2001; Tucker et al., 1999). The examination of essential fatty acids and the role of fish oil (associated with protein tissues) on bone mineral density has produced contradictory results, but some studies have suggested that it is beneficial by inhibiting the production of bone absorbing cells (osteoclasts), thereby increasing bone density (Fernandes, 2004). Finally, studies into vegetarian, vegan, and omnivorous diets have also presented conflicting results on the relationship between protein source and bone density, and several have been unable to detect differences in bone mineral density among people with different dietary preferences, suggesting that protein source may have no influence on bone density (Barr, 2004).

These conflicting results may reflect the methods used by modern studies. Relying on food frequency methods, 24 hour diet recall, and food history, clinical studies have usually been performed over a short period of time and less often longitudinally. Combined with measures of bone mineral density obtained through densitometric methods, such as dual x-ray absorptiometry, the nutritional documentation records are intended to provide information on the effects of diet on bone density; however, the downsides are the amount of time needed to collect this information and, the fact that participants often inaccurately estimate portion sizes and thus daily intakes of nutrients (Anderson, Switzer, Stewart, & Symons, 2004; Lau & Lau, 2004).

Bioarchaeologists can contribute to these studies through the use of stable isotope analysis. Stable isotope analysis can be used to directly investigate individual diet without the concerns of inaccurate reporting found in questionnaires. Because "you are what you eat", a record of past diet can be preserved in hard tissues such as bone, and may represent an approximately ten-to-thirty-year span of dietary information (Ambrose, 1993). This type of longitudinal study is an important contribution to the analysis of osteoporosis, a disease that occurs over years and not months, and can provide a window into the relationship between diet and bone density that clinical studies cannot. The advantage of bioarchaeological studies is that they allow for the study of individuals without some of the confounding factors that impact modern study populations, such as the widespread use of nutritional supplements and synthetic pharmaceuticals. In these ways, the use of

bioarchaeological methods can provide valuable insight into the etiology of osteoporosis in both living and past human populations.

This study focuses on aspects of diet that impact bone structure and may play a role in the development of osteoporosis. Bioarchaeological samples from medieval Portugal present a unique opportunity to examine the contributing factors that influence the etiology of osteoporosis. Direct dietary analysis of individuals using stable isotope analysis can be highly accurate, and when used in congruence with the analysis of bone mineral density, present insight into conflicting clinical studies.

This research project also has the potential to contribute to a number of fields. Contemporary research of osteoporosis will benefit from the historic perspective, which can further reveal the role nutrition plays in its manifestation. Archaeologists interested in medieval social patterns may also find this study of interest, as the comparative study of diet and disease can provide insight into socioeconomic relationships. The use of multiple lines of evidence such as historic records, archaeological evidence, and osteological analyses can offer additional perspective on the role of nutrition on bone health.

I will begin with a discussion of clinical studies on protein source and its relationship with bone mineral density. Chapter 2 will also discuss competing hypotheses, limitations of modern research, and how bioarchaeology may be used to address these issues. A review of bioarchaeological studies on osteoporosis is also presented. The chapter concludes with a review of how paleodietary stable isotope analysis of bone collagen may be used to test the competing clinical hypotheses.

In Chapter 3, I present a review of the historical and archaeological material, with a discussion of how this information might be used to further investigate the relationship between dietary protein source and bone mineral density. Chapter 4 provides information on the materials and methods used for this research project, including the determination of sex, age, disease status, and stable isotope values. In Chapter 5, I present the results of the current study, while Chapter 6 provides interpretation and discussion of all the material presented within this study. Finally, I will conclude by discussing the overall implications of this study and ideas for future analysis.

Chapter 2 OSTEOPOROSIS, EFFECTS OF DIET, AND METHODS OF EVALUATION

This chapter discusses the lack of consensus found in studies investigating the relationship between bone mineral density (BMD) (as a measure of osteoporosis) and sources of dietary protein, in addition to the hypotheses and goals of the current research project. I begin with an introduction to osteoporosis (OP) and its etiological factors, followed by a discussion of protein source and the clinical research that suggests four competing hypotheses with regard to its effect on BMD. I also discuss the limitations of modern research, as well as how bioarchaeological studies on BMD may present a different perspective on the study of OP. Finally, I review stable isotope analysis of paleodiets using bone collagen, and describe how stable isotope analysis may be used to test the four clinically derived hypotheses.

Osteoporosis is a skeletal disorder in which nutrition plays a key role in the pathology and treatment of the disease; various approaches have been used to investigate the complex relationship between diet and disease risk. Investigations of past and present human populations have examined the impact of nutritional intake on BMD (Agarwal & Glencross, 2010; Curate, 2005; Draper, 1994; Ferreira, 2012; Holick & Dawson-Hughes, 2004). Modern clinical studies have been unable to identify a clear relationship between dietary protein source and its effect on BMD. Studies have shown that animal and plant protein are metabolized differently, which can affect BMD; however, no consensus has been reached as to whether the effect of either protein source is positive, negative, or even neutral (Barr, 2004; Bonjour, Ammann, Chevalley, & Rizzoli, 2004; Bushinsky, 2004; Fernandes, 2004; Heaney & Layman, 2008; Pfeiffer & Lazenby, 1994; Sellmeyer et al., 2001).

2.1 Definitions of Osteoporosis

Osteoporosis is a multifactorial disease that affects both men and women that is associated with significant morbidity, mortality, and health care cost. Prentice (2004) states:

> The WHO definition of osteoporosis is a bone mineral content (BMC) or bone mineral density (BMD), measured by techniques such as dual-energy X-ray absorptiometry, that is more than 22.5 SD below the young adult mean for the population. A low BMC or density in an older person implies a sub-optimal bone mass in young adulthood (peak bone mass) or greater bone loss in later life, or both. (p. 227)

The disease is characterized by a decrease in bone density that can alter the microstructural architecture of bone. Increased skeletal fragility and thus, risk for fracture consequently occurs (Agarwal, 2008; Frost, 2003; Melton et al., 2003; Riggs, Kosla, & Melton, 1998, 2001; Rodan, Raisz, & Bilezikian, 2002). The clinical significance of OP lies in the occurrence of fracture. The risk of fractures increases progressively and continuously as BMD declines, and measurements of BMD are thus used to identify individuals at risk. The process by which BMD values can be used to diagnose individuals as suffering from OP or osteopenia will be discussed in more detail in Chapter 4.

Bone is composed of inorganic and organic intercellular matrix; the organic consisting mostly of the protein Type I collagen, which provides strength and flexibility (Katzenberg, 2008; Mays, 2000a; Schoeninger, 1995). As many researchers have pointed out, protein is essential for bone formation, and it accounts for approximately half of bone volume as it undergoes continuous turnover and remodeling (Heaney & Layman, 2008; Mays, 2000a).

Bone remodeling is the key process through which adult bone responds to mechanical and homeostatic influences, such as microfractures and calcium stasis (Matkovic, Badenhop-Stevens, Crncevic-Orlic, & Clairmont, 2004). Remodeling depends on the primary function of the bone being replaced, and bone tissue serving a metabolic function will be remodeled as part of its role in physiological homeostasis. However, the main function of bone is to resist mechanical loads, and over time this loading can cause an accumulation of damage (Agarwal, 2008). Damaged bone tissue is then remodeled to maintain or repair the skeleton. Bone loss associated with aging generally begins when the point of peak bone mass is reached, typically during the second and third decade of life (Currey, 2002; Rosen, 2004; Vaughn, 1981). According to Matkovic et al. (2004), "Peak bone mass is defined as the highest level of bone mass achieved as a result of normal growth (p. 174)." Therefore, peak bone mass is important because it sets the stage for bone health later in life; the propensity for fractures originates from the eventual loss of this peak mass. Generally, bone loss is occurs when bone resorption exceeds bone formation, resulting in unbalanced bone remodeling.

2.2 Causes of Osteoporosis

Osteoporosis is a complex disease. The broad factors underlying its development are well recognized; however, many of the specific mechanisms through which it becomes manifest remain unknown (Cummings & Melton, 2002). The principal factors for the onset of OP include increased age, menopause, mechanical loading, extreme exercise, peak bone mass and continuing sub-periosteal apposition, and genetics (Holick & Dawson-Hughes, 2004).

Hormonal changes associated with menopause are thought to be a leading cause of OP (Roberts & Manchester, 2005). The loss of estrogen that accompanies menopause can increase bone resorption by 90%, as compared to a 45% increase in bone formation. This ultimately results in a 5-10% loss of cortical bone and a 20-30% loss of trabecular bone (Pacifici, 2001; Raisz & Seeman, 2001; Riggs et al., 1998). The decrease of estrogen may also remove cell sensitivity to the strains of physical activity, further contributing to the atrophy of bone tissue (Frost, 2003; Raisz & Seeman, 2001; Riggs et al., 2001).

Osteoporosis-related fractures are more frequent in post-menopausal women than among men of a similar age, demonstrating the impact of menopause on skeletal health (Kanis, 1994; Pacifici, 2001; Riggs, et al., 2001). However, not all post-menopausal women will develop OP. The onset of menopause, coupled with individual factors that may include poor nutrition or genetic background, increases the rate of bone loss and risk for fracture (Riggs et al., 1998, 2001).

As discussed by Brickley and Ives (2008), a number of other processes can contribute towards the development of OP. These are injuries or pathologies (e.g., fracture, stroke) that may lead to subsequent anatomical disuse (e.g., decreased mobility) causing skeletal atrophy, the consequence of a disease (e.g., leprosy, tuberculosis), and nutritional deficits that disrupt mineral metabolism.

Most relevant to this study is the relationship between OP and diet. Diet affects OP because it impacts mineral homeostasis, collagen production, and bone turnover rates. The effects of dietary macronutrients, minerals, and micronutrients on bone health have been studied extensively in clinical settings; these studies have illustrated the role that diet can play in the pathogenesis of osteoporosis (Holick & Dawson-Hughes, 2004).

Calcium absorption is critical to bone health and a sufficient amount of calcium intake during adolescence can help optimize peak bone mass (Miller, Jarvis, & McBean, 2001). Because 99% of the body's calcium is found in the skeleton, it is a vital element that plays an important role in the pathogenesis of osteoporosis (Heaney, 1997). Dietary calcium intake must remain greater than calcium loss to maintain a healthy skeletal supply and thus calcium intake needs to be balanced by good intestinal absorption as well as renal excretion and reabsorption. Without the proper intake of calcium, or its limited absorption, bone is resorbed to buffer the calcium deficit, contributing to the occurrence of OP (Heaney, 1997).

Calcium absorption can also be affected by foods that generate a net acidifying or alkalizing effect during metabolism. During fluxes of metabolic acidosis, pH levels are reduced, osteoclastic

activity is stimulated, and bone is resorbed. This releases calcium, thereby rebalancing the pH level (Bushinsky, 2004). Chronic metabolic acidosis increases urinary calcium excretion without an increase in intestinal calcium absorption, which ultimately results in loss of bone mineral (Barzel, 1970, 1976; Barzel, 1995 as cited in Bushinsky, 2004). The effects of acidosis have been demonstrated in children suffering from renal tubular acidosis, as well as adults with distal renal tubular acidosis; however, administration of a basic substance like bicarbonate can decrease the negative calcium balance and result in an increase in bone mineral density (Domrongkitchaiporn et al., 2002 as cited in Bushinsky, 2004; McSherry & Morris, 1978).

The role played by fatty acids in the development of OP has been a focus of recent research. Essential fatty acids (EFAs) are obtained mainly from plants or marine sources (e.g., fish) and are of critical importance because the body cannot synthesize these fatty acids. Increasing clinical evidence shows that fatty acid deficiency may contribute to bone loss (Das, 2000; Kruger & Horrobin, 1997). Animal and human studies on the influence of fatty acids on bone metabolism have revealed that a decrease in the *n*-6/*n*-3 fatty acid ratio can protect against bone mineral loss (Fernandes, 2004; Wohl, Loehrke, Watkins, & Zernicke, 1998). The mechanism by which these fatty acids inhibit bone loss has not been determined, although it has been proposed that its protective action may be a result of a decrease in urinary calcium loss, thus preventing metabolic acidosis (Claassen, Coetzer, Steinmann, & Kruger, 1995; Kruger, Coetzer, De Winter, Gericke, & van Papendorp, 1998). Moreover, diets high in saturated fat (e.g., animal fat products) have also been reported to have a detrimental effect on bone metabolism due their interference with calcium metabolism and absorption (Brickley & Ives, 2008; Fernandes, 2004).

As discussed in Chapter 1, the role of dietary protein in OP has been a point of controversy ever since the endogenous acid hypothesis was proposed by Wachman and Bernstein (1968). Their hypothesis stated that protein-rich diets increase bone loss due to metabolic acidosis and the consequent need for calcium resorption from the skeleton, buffering the elevated acidity. Protein has been identified as being both detrimental and beneficial to bone health depending on a variety of factors: the level of protein in the diet, the protein source, calcium intake, and the acid/base balance of the diet (Heaney & Layman, 2008). Heaney and Layman (2008) state that:

> Protein intake affects bone in several ways: 1) it provides the structural matrix of the bone, 2) it optimizes [Insulin-like Growth Factor] IGF-1 levels, 3) it is reported to

increase urinary calcium, and 4) it is reported to increase intestinal calcium absorption. (p. 1567S)

Several studies have investigated the effect of animal-based versus plant-based proteins on bone metabolism, but their results have been contradictory. Some, for instance, have found that meat as a protein source may be associated with increased bone mineralization (Bonjour et al., 2004; Rosen, 2004; Takata, Maskarinec, Rinaldi, Kaaks, & Nagata, 2006). This is because animal protein is associated with higher serum levels of Insulin-like Growth Factor-I (IGF-I), and IGF-I plays a key role in bone metabolism, where higher levels of it are osteotrophic. As people age, there is also a decline in serum concentrations that adversely influences bone remodeling (Heaney & Layman, 2008; Rosen, 2004). In contrast, soy (a legume) as a protein source has been linked with low levels of IGF-1 (Takata et al., 2006).

Conversely, others have proposed that diets high in animal protein may have a greater negative effect on BMD than diets high in vegetable protein because animal protein induces a greater increase in urinary calcium excretion (Hu, Zhao, Parpia, & Campbell, 1993; Sellmeyer et al., 2001; Wachman & Bernstein, 1968). A study by Hu and coworkers (1993) found that urinary excretion of calcium was positively correlated with the consumption of animal protein in middle-aged and elderly women in China. However, the Framingham Osteoporosis Study produced opposite results, showing that elderly men and women who consumed animal protein did not demonstrate a decrease in BMD (Hannan et al., 2000).

Studies performed with ovariectomized lab mice have shown that fish oil consumption may support bone health (Fernandes, 2004; Fernandes, Lawrence, & Sun, 2003; Fernandes, Sun, Krishnan, & Zaman, 2002; Zalloua et al., 2007). By inhibiting osteoclast progenitors, the fatty acids in fish oil have been shown to inhibit osteoclastogenesis – this possibly being the primary mechanism of the protective effect of fish oil on BMD (Fernandes, 2004). Although oils are not proteins, they are intimately associated with the muscle protein consumed by humans and *n*-3 fatty acids have been reported to protect against bone loss (Kruger et al., 1998). However, any mechanism by which bone density is increased must be accompanied by adequate intake of the necessary constituents for bone formation, including calcium and phosphorous (Heaney, 2001). With this in mind, Fernandes (2004) suggests that a combination of fish oil and isoflavone sources of plant protein (like those found in soy) may be healthier for bone metabolism when compared to

animal protein; where the phytoestrogens in legumes, such as soy, aid in the decrease of bone loss brought on by estrogen deficiency (Fernandes, 2004).

Studies of vegetarian, vegan, and omnivorous diets have presented conflicting results on the relationship between diet and bone density (Barr, Prior, Janelle, & Lentle, 1998; Ellis, Holesh, & Ellis, 1972; Marsh, Sanchez, Mickelsen, Keiser, & Mayor, 1980; Outila, Karkkainen, Seppanen, & Lamber-Allardt, 2000). Most vegetarian and/or vegan diets contain different sources of protein from those of omnivorous diets, and long-term adherence to a vegan diet appears to be consistently associated with lower BMD (Barr, 2004). Nevertheless, several studies have been unable to detect differences in BMD between vegetarian and omnivorous postmenopausal women, suggesting protein source has no influence on BMD (Barr, 2004).

Essentially, four competing hypotheses regarding the relationship between protein source and OP can be derived from these studies: 1) animal meat consumption increases bone loss, resulting in decreased bone density, 2) meat consumption has a positive effect on bone density by increasing bone mineralization, 3) fatty acids found in seafood, specifically fish oil, have a beneficial effect on bone health by decreasing bone loss, and lastly, 4) protein source has no influence on BMD.

2.3 Clinical Limitations and Bioarchaeological Benefits

Clinical studies on the relationship between nutrition and bone health are based on a variety of methods for capturing dietary intakes, including assessment of overall nutritional status, food-frequency methods, twenty-four hour diet recall, food diaries (or record), and food history (Lau & Lau, 2004). These studies may be performed over a short period of time or part of longitudinal studies. The majority of these nutritional documentation studies are combined with analyses of bone mineral density (BMD) obtained through densitometric devices (e.g., dual x-ray absorptiometry, quantitative computed tomography).

The food frequency method has been the most frequently applied in epidemiological studies on nutrition and bone health. It is a questionnaire used to assess nutrition in light of bone health over weeks, months, or years; the concept being that long-term intake of nutrients has more implications on bone health during stages of remodeling (Lau & Lau, 2004; Raisz, 2004). The basic food-frequency questionnaire consists of two components: a food list and a frequency response section to evaluate how often the food is eaten (Anderson et al., 2004; Lau & Lau, 2004). It may be assessed qualitatively or quantitatively, and studies may incorporate thousands of participants

(Anderson et al., 2004). Qualitative food frequency assessment may underestimate overall energy intake for a few days but can rank individuals appropriately for comparisons between or among groups (Anderson et al., 2004). Quantitative food frequency questionnaires are the most frequently used because they are able to capture nutrition intake within the context of a longer time frame; however, the downside is the amount of time needed to collect this information, in addition to the fact that participants often inaccurately estimate daily intakes of nutrients (Anderson et al., 2004; Lau & Lau 2004).

In OP structural changes in cortical and trabecular bone can be distinguished either radiologically or histologically (Brickley & Ives, 2008). Cortical bone has been observed to thin by an increase in intra-cortical porosity and an increase in endosteal resorption leading to a coalescence of resorption cavities and bone loss. Additionally, there is an increase in the formation of trabecular bone as medullary space is increased. Fatigue damage (micro-fracturing) is also accumulated and increased as bone remodeling slows (Cho, Stout, & Bishop, 2006; Compston, 1999; Frost, Fogelman, Blake, Marsden, & Cook, 2004; Kanis, 1994; Parfitt, 1986, 1994; Recker & Barger-Lux, 2001). Affected trabecular bone is recognized by perforated trabeculae, the presence of accumulated micro-fractures, reduced strut length, and reduced trabecular connectivity. In compensation, the thickening of remaining trabeculae may manifest, but the resorption of trabecular surfaces may also occur as a way to reduce the need for future remodeling - hence the overall thinning of trabecular bone (Banse et al., 2003; Brickley & Howell, 1999; Compston, 1999; Frost et al., 2004; Marcus & Majumder, 2001; Melton, Chao, & Lane, 1988; Roberts & Wakely, 1992).

Today, clinical diagnosis of OP is accomplished via a number of techniques that measure bone mineral density (BMD). These are commonly divided into central and peripheral methods. Central methods obtain measurements of the spine and proximal femur by means of dual x-ray absorptiometry (DXA) and quantitative computed tomography (QCT); peripheral methods obtain measurements from the phalanges, forearm, and/or calcaneus with the use of peripheral dual x-ray absorptiometry (pDXA) and peripheral quantitative computed tomography (pQCT). Although it does not measure BMD, quantitative ultrasound (QUS) is also regularly included in peripheral methods of diagnosis (Lenchik et al., 2004). These methods provide either an areal or volumetric measurement of BMD and are used to assess fracture risk in both cortical and trabecular bone depending on region of interest (Lenchik et al., 2004).

Clinical diagnosis and the definition of OP has changed over time and varied between studies. While the definitive feature of OP used to be the identification of fracture from one or more

regions of interest (vertebrae, femoral neck, and/or distal radius), measurement of BMD analysis allows for the identification of risk before fracture occurs. Thus, the clinical assessment of bone mineral density and the diagnosis of bone loss is done by non-invasive *in vivo* methods generally achieved through densitometry methods that are based on the differential absorption of radiation by bone and soft tissue.

Bioarchaeologists employ three methods to diagnose OP in past populations: radiology, histology, and visual examination. Archaeological bone can be studied at the gross and microscopic level, and a variety of methods have been employed to identify OP among archaeological populations (Brickley & Agarwal, 2003). In general, though, there is an impetus to apply clinical methods to archaeological remains in order to facilitate comparisons by different researchers. For a complete overview of the methods employed by bioarchaeologists to investigate bone density see Brickley and Agarwal (2003).

The investigation of OP and BMD among past populations with vastly different diets and lifestyles from those seen in modern, post-industrial populations are beneficial in the study of a multifactorial disease such as OP. The advantage of bioarchaeological studies is that they allow for the study of individuals with fewer confounding factors, such as the use of pharmaceuticals. In addition, the analysis of bone provides an archive of long-term diet that is superior to modern studies that rely upon short-term, error-prone food questionnaires. The use of bioarchaeological methods contributes to our understanding of OP in the past and provides valuable insight into the etiology of osteoporosis in both living and past human populations.

2.4 Osteoporosis from an Anthropological Perspective

Osteoporosis has been of interest to biological anthropologists for years in part because of its importance as a modern health concern. By seeking out patterns and prevalence of OP in the past, anthropologists can contribute to our broader understanding of the disease, through consideration of such aspects of bone maintenance as growth, nutrition, activity, and reproductive status (Agarwal, 2008).

The relationship between mechanical use (and activity patterns) and bone fragility has been examined among archaeological populations. Aspects of lifestyle, such as workload, underwent changes throughout prehistory, and comparisons of agricultural and hunter-gatherer skeletal populations suggest that a decline in activity with domestication resulted in an increased prevalence of OP (Larsen, 1997, 2003; Ruff, Larsen, Hayes, 1984).

Paleopathological studies of nutrition and bone loss are also common but have assessed the impact of nutrition primarily through the comparison of groups with different subsistence economies. Erickson (1976) compared radiographic data from the femora and humeri across three different populations that varied in subsistence sources: primarily animal meat subsistence (Alaskan Eskimo), mixed hunting and farming (Arikara), and maize agriculture (Pueblo). All groups were found to lose bone mass as they aged, with females exhibiting a greater magnitude and rates of loss than males; however, the most BMD loss occurred in individuals belonging to the Puebloan sample population. This discovery led Erickson (1976) to hypothesize that a diet consisting mainly of maize was detrimental to skeletal health.

The effects of animal protein on BMD have also been studied among prehistoric populations, mainly those in the Arctic due to their animal protein-heavy diets. Multiple studies have found that Eskimo and Inuit populations show thinner cortices and lower bone mineral index when compared to similarly aged Caucasian populations, leading some to suggest that animal protein is detrimental to BMD (Thompson & Guiness-Hey, 1981; Thompson, Slater, & Laughlin, 1981; Thompson, Slater, Laughlin, & Blumenthal, 1983; Thompson & Cowen, 1984). A more detailed review of paleopathological studies of bone loss and nutrition can be found in Curate (in press).

To a limited degree, stable isotope analysis has also been utilized to examine the relationship between diet and OP. White and Armelagos (1997) demonstrated the potential of stable isotope analysis in a study of osteopenia (precursor to OP) and found marked differences in the nitrogen stable isotope ratio of bone collagen between normal and osteopenic females from a single population. Their analysis of 43 Sudanese Nubians from the X-Group period suggested physiological factors (e.g., water stress) were the predominant causative factor of osteopenia. They rejected a dietary explanation for the difference in nitrogen values in osteopenic females, stating that the high $\delta^{15}N‰$ values may be reflective of urea excretion and therefore kidney function – both occurring in lactating mothers and menopausal women where dehydration may be a factor – rather than reflecting protein intake. "The northern Sudan, both past and present, does not support the kind of substantial animal production which would sustain the degree of high protein consumption needed to create osteopenia." (White & Armelagos, 1997, p. 189).

However, a subsequent study by Thompson et al. (2008) found that the Sudanese human $\delta^{15}N$ values are elevated over fauna, suggesting significant animal protein consumption. Even though humans and animals may experience water stress differently, this offers a contradictory

explanation to White and Armelagos' (1997) water stress account and proposes that diet may have contributed to elevated nitrogen stable isotope ratios.

The examination of past populations can contribute to our understanding of the role nutrition plays in skeletal growth and maintenance – in addition to illustrating its synergism with other biological, social, and cultural influences on the skeleton. Additionally, utilizing stable isotope analysis allows bioarchaeologists to directly investigate dietary patterns.

2.5 Stable Isotope Analysis in Bioarchaeology

Stable isotope analysis allows for the reconstruction of individual diets from past populations (Katzenberg, 2008; Schoeninger, 2011). Using stable isotope analysis to reconstruct diet requires some basic knowledge of atomic chemistry. Most elements exist as two or more isotopes; some isotopes are radioactive and steadily decay while others do not and are stable (Schoeninger, 1995; Schoeninger & Moore, 1992). One must understand that isotopes are atoms of the same element that have different masses due to different numbers of neutrons; these small mass differences translate into differences in rates of reaction with the lighter isotopes reacting more rapidly than the heavier isotopes (Katzenberg, 2008; Schoeninger, 1995; Schoeninger & Moore, 1992). This difference in rate of reaction leads to isotope sorting or 'fractionation' during chemical and physical processes, leading to differences in the ratios of heavy and light isotopes in substances on Earth (Koch 2007, Mays 2000b). More detailed information on isotope effects and fractionation can be found in Hoefs (1997) and Fry (2006).

Stable isotope analysis of paleodiet is essentially based on the old adage "you are what you eat." Stable isotope ratios of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) are particularly useful because they vary in major classes of foodstuffs, and those variations are passed on to consumers. This dietary signature persists in the skeletal remains. As a hard tissue, bone survives well in the archaeological record and is the most frequently used material for paleodietary reconstruction. Because bone collagen remodels slowly throughout life, and is renewed and replaced over a period of 10-30 years (depending on the bone and the individual), it provides a long-term average of diet (Ambrose, 1993).

Bone collagen, the most abundant protein within bone, is considered to reflect mainly the protein portion of the diet rather than diet as a whole (Ambrose, 1990, 1993; Katzenberg, 2008). Although the carbon from collagen comes mainly from protein, it may be contributed in small portions by other macronutrients (carbohydrates and lipids), especially if dietary protein intake is

low. Effectively, all nitrogen in diet comes from protein; thus, $\delta^{15}N$ values reflect dietary protein (Mays, 2000a; Schoeninger, 1995).

Isotope ratios are determined with the use of a mass spectrometer and are expressed in delta notation in parts per mil relative to a standard (Schoeninger & Moore, 1992). The international standard for carbon is Vienna PeeDee Belemnite Carbonate (VPDB), a marine carbonate; for nitrogen, the sample ratio is compared relative to AIR (ambient inhalable reserve) (Katzenberg, 2008; Schoeninger & Moore, 1992). The isotope ratios are calculated as follows (Equation 1 carbon isotope ratio calculation and Equation 2- nitrogen isotope ratio calculation, respectively):

$$\left[\delta^{13}C = \frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{VPDBstandard}} - 1\right] \times 1000 \quad [1]$$

$$\left[\delta^{15}N = \frac{^{15}N/^{14}N_{sample}}{^{15}N/^{14}N_{AIRstandard}} - 1\right] \times 1000 \quad [2]$$

Because most biological materials contain less ^{13}C relative to ^{12}C than the standards, $\delta^{13}C$ values are generally negative. Additionally, since the majority of biological tissues contain more ^{15}N than air (which is zero) the $\delta^{15}N$ is generally greater than zero (Mays, 2000b; Schoeninger, 1995).

2.6 Carbon Isotope Ratios and Diet

There are two stable isotopes of carbon, ^{13}C and ^{12}C, which appear in the environment with natural abundances of approximately 1.1% and 98.9%, respectively (Hoefs, 1997; Schoeninger, 1990). Carbon isotope ratios are used to estimate the proportional intake of protein from C3 vs. C4 resources and from marine vs. terrestrial resources (Chisholm et al., 1982; Katzenberg, 2008). Moreover, "[t]he $\delta^{13}C$ values of animal collagen reflect the isotopic compositions of plants at the base of the food chain in an ecosystem" (Koch, Fogel, & Tuross, 1994, p. 68).

Because the atmosphere serves as terrestrial plants' major source of CO_2, this, in combination with the plant's photosynthetic pathway, will determine the plant's $\delta^{13}C$ (Schoeninger & Moore, 1992). Plants using the C3 photosynthetic pathway (such as wheat, barley, other temperate grasses, and shrubs and trees) exhibit more negative $\delta^{13}C$ values than plants using the C4 photosynthetic pathway (such as millet, sorghum, maize, and other tropical grasses) (DeNiro, 1987; Katzenberg, 2008; Schoeninger & Moore, 1992). C3 plants have $\delta^{13}C$ values between -20 and -35‰

with most species at about -26‰ (Katzenberg, 2008; Schoeninger & Moore, 1992). C4 plants have $\delta^{13}C$ values ranging from -9 to -16‰ (Katzenberg, 2008; Schoeninger & Moore, 1992). As Katzenberg (2008) states so succinctly, "the non-overlapping ranges of C3 and C4 plants provide the basis for using stable isotopes of carbon in preserved human tissue for revealing diet (p. 424)"

Carbon isotope ratios measured on consumer tissues can also reveal the importance of marine versus terrestrial foods in the diet. Carbon isotope values of marine foods typically fall in between those of C3 and C4 plants (Schoeninger, 1995). In the absence of control data, it is assumed that individuals who consumed a mixture of marine and terrestrial foods would have bone collagen $\delta^{13}C$ values linearly scaled between the low terrestrial end-point (~-20‰) and the high marine end-point (~-15‰) (Chisholm, Nelson, & Schwarcz, 1982; Katzenberg, 2008; Lubell & Jackes, 1994). Freshwater systems can be more difficult to assess since their $\delta^{13}C$ values are derived from a mixture of carbon from terrestrial detritus, dissolved atmospheric CO_2, and reaction with limestone bedrock (where applicable). These values will then reflect the relative contribution of each source and may vary widely (Schwarcz & Schoeninger, 1991).

Where both marine foods and C4 plants (i.e., millet) may have been consumed, the use of carbon stable isotopes alone to determine dietary components is insufficient. Although the isotopic ratio of bone collagen reflects the protein component of the diet and carbon can aid in the distinction of terrestrial versus marine dietary protein to some degree, marine organisms will have an isotopic signal that is intermediate between terrestrial C3 and C4 values, essentially blurring the lines of interpretation from $\delta^{13}C$ values alone (Schoeninger, 1995). The use of nitrogen isotope ratios to distinguish terrestrial and marine dietary protein sources is discussed below.

2.7 Nitrogen Isotope Ratios and Diet

Nitrogen also comes in the form of two stable isotopes, ^{15}N and ^{14}N – with a natural abundance ratio of 0.36 and 99.64%, respectively (Hoefs, 1997; Schoeninger & Moore, 1992; Schwarcz & Schoeninger, 1991). Two major processes transfer nitrogen into the biological realm: bacterial degradation and nitrogen-fixing organisms. The majority of terrestrial plants take up nitrogen from the soil – during decomposition, bacteria break down organisms, producing nitrates that vascular plants can use directly to produce a $\delta^{15}N$ value more positive than the atmosphere (Schoeninger & Moore, 1992; Schwarcz & Schoeninger, 1991). These tissues have $\delta^{15}N$ values close to zero reflecting the atmospheric N_2 content (Schoeninger & Moore, 1992).

The $\delta^{15}N$ values in the tissue of consumers are positively correlated with the values in their diets and there is a gradient increase in delta values from one trophic level to the next. A stepwise increase in $\delta^{15}N$ values occurs with each trophic level, with a minimal 3‰ increase (Katzenberg, 2008; Schwarcz & Schoeninger, 1991). The enrichment of nitrogen isotope ratios with every step in the food chain occurs because more ^{14}N is excreted by the individual in urea, leaving more ^{15}N behind for physiological use. During metabolism, the bonds between the lighter isotopes (^{14}N) are broken more easily than those between the heavier, and thereby more readily shed from the system (Schoeninger & Moore, 1992). This spacing between the diet and consumer can be applied to both vertebrates and invertebrates (Minigawa & Wada, 1984; Schoeninger & DeNiro, 1984). Therefore, $\delta^{15}N$ values in bone collagen can be used to identify herbivores, omnivores, and carnivores, in both marine and terrestrial ecosystems (Ambrose, Butler, Hanson, Hunter-Anderson, & Krueger, 1997; Katzenberg, 2008).

Nitrogen isotope values can also be used to distinguish consumers of marine or terrestrial foods. Marine organisms tend to have a more positive $\delta^{15}N$ value than terrestrial organisms due in part to the bacterial and algal fixation processes that produce N-containing compounds with higher $\delta^{15}N$ values. Similarly, freshwater systems may have an increased $\delta^{15}N$ value over terrestrial organisms as well (Katzenberg, 2008; Schwarcz & Schoeninger, 1991). Thus, even though great variability exists in marine $\delta^{15}N$ values, the majority are elevated over terrestrial organisms (Schoeninger & Moore, 1992). Marine vertebrates have $\delta^{15}N$ values that may be as much as 8‰ more positive than terrestrial vertebrates at a similar trophic position, due to the larger range of trophic levels found in marine environments (Schoeninger, 1995).

It is important to understand that $\delta^{15}N$ values from bone collagen alone cannot be used to estimate the *amount* of meat in overall human diet. This is because collagen does not characterize whole diet (Schwarcz & Schoeninger, 1991). It is not possible to distinguish between an individual who eats one pound of meat and another who eats two pounds. Because meat is extremely high in protein in comparison to plants, a relatively small amount of meat in a diet has a large effect on the consumer and their $\delta^{15}N$ value (van Klinken, Richards, & Hedges, 2000).

2.8 Caveats in Carbon and Nitrogen Isotope Ratios and Diet Reconstruction

Confounding factors in the use of stable isotope ratios to reconstruct diet include but are not limited to water stress, manuring, and geographic variability. These can cause variability among regions, ecosystems, and even cultures.

Water stress is an aspect to consider when investigating humans and animals from arid regions. Under conditions of water stress, more urea is excreted relative to the volume of urine retaining more ^{15}N in the body resulting in bodily tissue that is increased in $\delta^{15}N$ (Katzenberg, 2008). Inaccurate dietary interpretations can result if higher $\delta^{15}N$ values are attributed to the use of marine protein sources instead of recognizing that the higher values may be due to water stress. Generally, the effect of water stress on N^{15} levels and its role on individual nitrogen isotope ratios is complex (Ambrose, 1991, 2001; Ambrose & DeNiro, 1986). For a review on water stress see Schwarcz and Schoeninger (1991, p. 299-300).

Studies into manuring practices in the cultivation of cereals and grains suggest that the practice can result in increased $\delta^{15}N$ values in crops. This resulting enrichment of ^{15}N in plants would yield values in consumers resembling either a mixed plant-and-animal-based diet or a largely animal-based diet (Bogaard, Heaton, Poulton, & Merbach, 2007). Interpretation of human nitrogen isotope values that appear one trophic level higher than associated herbivores are regarded as reflecting largely animal-based diets. The cultural practice of manuring crops could provide an alternative explanation for this pattern, reflecting consumption of grain that has been enriched in ^{15}N at the base of the food web (Bogaard et al., 2007).

Stable isotope values change with time and vary geographically – environmental factors such as variations in the atmospheric content of CO_2 and N_2 and climate all affect isotopic variability in plants, ultimately determining the isotopic signature in the food eaten by humans (van Klinken et al., 2000). Spatial variations include differences in marine and continental air both of which vary with latitude and season. The canopy effect is an example of spatial variation and occurs in dense vegetation where plants respire CO_2, causing a systematic bias between plants and animals residing within forests vs. open environments (van Klinken et al., 2000). Influences of temperature and/or relative humidity also cause regional patterning effects on plant photosynthesis, influencing their isotopic compositions and thereby reflecting regional food web ecosystems (van Klinken et al., 2000).

Many of these confounding factors can be circumvented by the comparison of data from botanical and faunal remains contemporary to those remains of interest (e.g., human remains). The need to assess environmental and anthropogenic effects in the ecological food web is important when reconstructing paleodiet. Without the measurement of associated plant and animal remains, robust dietary reconstructions are not possible.

2.9 Diet Modeling Using Stable Isotope Analysis

All diet models are based on the premise that the isotopic composition of consumer tissues reflects the isotopic composition of the food sources, after adjusting for known offsets between diet and tissue. The isotopic composition of consumer tissues reflects but is not identical to that of the diet. For carbon ratios there is a small offset between diet and the whole body of the consumer, but individual tissues may have larger offsets. The estimated offset between $\delta^{13}C$ in diet and bone collagen is around 5‰; however, this can vary depending on the difference between the $\delta^{13}C$ value of the protein source and the whole diet (Ambrose et al., 1997). A reasonable estimate for the fractionation of carbon between diet and collagen is 3 - 5‰ (Schoeninger & Moore, 1992).

The difference in $\delta^{15}N$ values between diet and bone collagen indicate an approximate 3‰ enrichment over diet, this being the commonly used value (Schoeninger & Moore, 1992). However, there are studies that contradict this and show that collagen may be enriched far more than 3‰ (Bocherens & Drucker, 2003), reasons for this may have to do with agronomic and physiological variation (for further discussion see Hedges & Reynard, 2007; Schoeninger & Moore, 1992). Importantly, comparisons across environmental systems should not be attempted since nitrogen isotope composition varies and is not necessarily equivalent in all areas (van Klinken et al., 2000). Nevertheless, the minimum 3‰ enrichment between trophic levels seems to hold constant.

Diet reconstruction with stable isotopes involves the comparison of stable isotope ratios measured on consumers to those of their potential food sources. Such comparisons may include simple visual analysis of isotope plots or advanced mixing models that estimate the proportional contributions of various foodstuffs using mathematical equations. Often, these methods will include information from archaeobotanical, zooarchaeological, and (if available) ethnographic evidence.

Dietary mixing models consist of mathematical equations that attempt to explain the consumers' isotopic composition as a mixture of its dietary isotopic composition (Phillips, 2012). Dietary modeling can also be undertaken without the use of mixing models and is a common method for bioarchaeologists to employ even when detailed information is available regarding food source isotope ratios. These studies often consist of inferences based on a comparison of consumer and food source stable isotope ratios established by visual analysis of isotope ratio plots. Some knowledge of food sources and the environment is critical for these studies, and the sources must have isotopically distinct signatures (e.g., terrestrial vs. marine food sources) (Phillips, 2012). This way, a bivariate plot of $\delta^{13}C$ and $\delta^{15}N$ values can provide general inferences on the isotopic niche of individuals (Layman et al., 2012).

As previously mentioned, carbon values often overlap in marine versus terrestrial protein sources (Ambrose, 1993; Katzenberg, 2008). Because nitrogen also reflects dietary protein sources, its analysis in congruence with carbon can aid in the interpretation of the consumption dietary marine protein. Marine protein intake can be estimated using a linear regression of both $\delta^{13}C$ and $\delta^{15}N$ values. Additionally, $\delta^{15}N$ values also aid in defining the trophic level of the protein consumed (Figure 1) (Richards & Hedges, 1999).

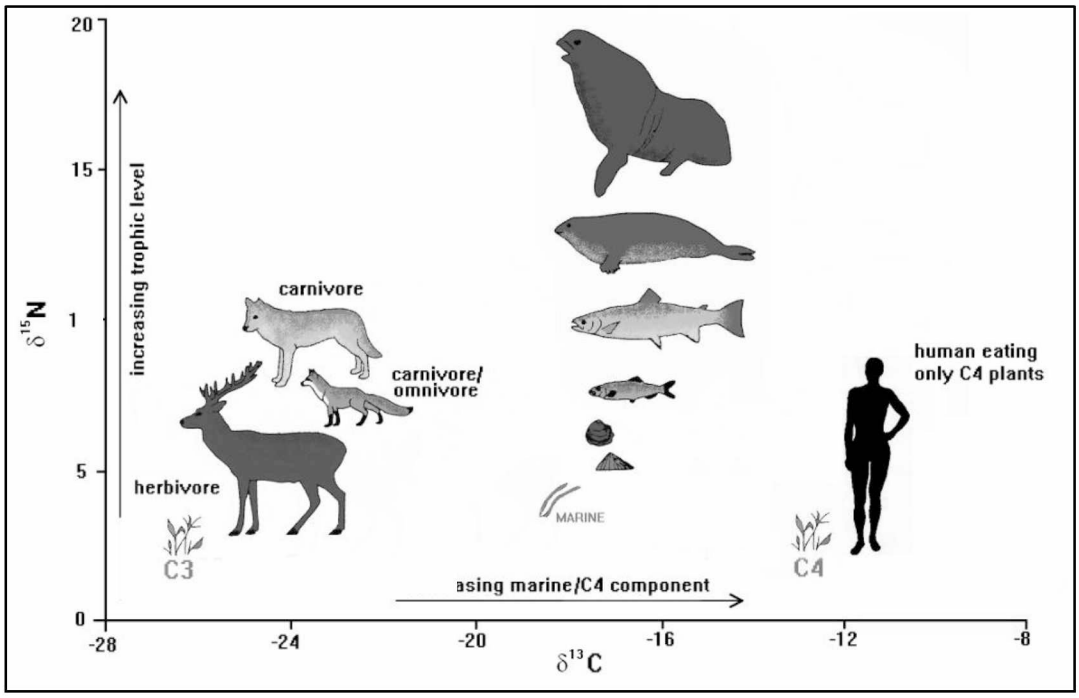

Figure 1. Simplified summary of stable carbon and nitrogen values for terrestrial and marine ecosystems. (Modified from Schulting, 1998).

2.10 Stable Isotope Analysis and Bone Mineral Density

Carbon and nitrogen stable isotope measurements on bone collagen can reveal the relative contributions of various dietary protein sources that may affect BMD, including animal vs. plant protein and marine vs. terrestrial protein (Figure 1). The four competing hypothesis regarding the effect of dietary protein source on BMD can be investigated using stable isotope analysis, with clear expectations for the relationship between BMD and stable isotope ratios.

In summary, the hypotheses for this study are 1) that a high ratio of animal to vegetable intake will increase bone loss and risk of fracture. If this is the case, I expect to find a *negative* relationship between bone mineral density values and nitrogen isotope ratios; 2) that a high ratio of animal to vegetable intake is associated with an increase in bone mineral density. In this case, bone mineral density values and nitrogen isotope ratios should exhibit a *positive* linear association; 3) that the consumption of seafood is associated with an increase in bone mineral density. In this case, there should be a *positive* linear association between bone mineral density for both nitrogen and carbon isotope ratios; and lastly, 4) that protein source has no significant effect on bone mineral density, and thus I would expect to find no linear relationship between variables, either positive or negative.

Exploration of these relationships will be done using a sample of individuals from three regions of medieval Portugal. This research project has been conducted in collaboration with the University of Coimbra, Portugal. The remains utilized in this study were previously assessed for BMD and the presence of OP (Curate, personal communication, August 12, 2014; Ferreira, 2012). The samples derive from three different regions, providing the opportunity to compare and contrast diverging dietary regimes within a contemporaneous time frame (13^{th} to 16^{th} century). Details on the samples available for this study, their context, and their analyses are discussed in the following chapters. The next chapter presents the historical and archaeological background of the samples and provides context for the individuals studied.

Chapter 3 HISTORICAL AND ARCHAEOLOGICAL CONTEXT

The samples for this project come from the Portuguese cities of Coimbra, Santarém, and Cacela Velha; while most of the samples date to the medieval period, a few may derive from the Renaissance (Figure 2 and Figure 3) (Livermore, 1973; Marques, 1971). The medieval period in Portugal is broadly considered to have begun in the 5th century AD and ended with the taking of Constantinople in the beginning of the 15th century (Ferreira, 2012). The Renaissance dates to the 15th and 17th centuries and although this period marks a time of great changes in Portuguese culture and arts, many of the same foods were still being consumed. Because this study focuses on the medieval period, specifically the 13th – 16th century, the inclusion of a few individuals from the early Renaissance era should not affect the outcome (Livermore, 1973; Marques 1971).

Figure 2. Map of the Iberian Peninsula showing traditional provinces and cities. Sites from this study indicated by stars. (Modified map from Livermore, 1973).

Figure 3. Map of Iberian Peninsula illustrating sites of interest: São João de Almedina in Coimbra, Convento de São Francisco in Santarém, and the necropolis of Cacela at Cacela Velha.

The county of Portugal (1093 – 1139AD) became a consolidated kingdom after it gained independence from the Kingdom of León under the acclaimed king, Afonso Henriques (Livermore, 1973; O'Callaghan, 1975). Defeat over the Moors in 1249AD, and subsequent reclamation of the southern region known as the Algarve, solidified Portugal's boundaries that have remained essentially unchanged since the 13th century. Although medieval Portugal was a period of transformation both socially and economically, trade thrived for all classes and religions during the reign of Dinis (1279-1325AD), the sixth king of Portugal. Nicknamed "the Farmer King," King Dinis took special interest in encouraging forestry plantation and development of the country's agricultural resources. Additionally, he showed concern for maritime exploration and the extension and protection of commerce (Livermore, 1973; O'Callaghan, 1975). This period of growth did not go unchecked, and in the mid-14th century, as with the rest of Europe, Portugal was devastated by

the Black Death. Increased strife occurred following the death of King Fernando (1367-1383AD) when Portugal rose in rebellion for two years fighting to remain independent from Castile. The kingdom's situation took a turn for the better during the 15th century when Portugal became known as a great maritime nation; Prince Henrique "the Navigator" (1394-1460) provided funding and ships to Portuguese captains to venture further afield providing new territories for Portugal to rule. During the 16th century Portugal profited from the spice trade, saw the advent of the Inquisition, and colonized Brazil (Livermore, 1973; O'Callaghan, 1975).

Each of the three sites in this study has a unique background. Cacela-a-Velha was used as a burial site during the 13th and 14th centuries and radiocarbon analysis from two individuals buried at Cacela-a-Velha (Cacela) returned calibrated dates of 1240 AD and 1260 AD (Curate, in press; Ferreira, 2012). The Convento São Francisco (Convent of SF) was founded in 1242 AD, and while this burial ground was in use for five centuries after its inception, epitaphs may place a few of the interments during the 16th century (Ferreira, 2012; Ramalho, 2001). The church of São João de Almedina (SJ de Almedina) was in use from the end of the 12th century until the late 15th century; however, no documentation on the archaeological excavations is available so we cannot narrow the date range any further (Ferreira, 2012).

Many of the Portuguese texts referenced here were translated with the use of Google Translate. Dr. Francisco Curate also occasionally aided in translation. Any omissions or erroneous information are the author's and reflect the limits of technologically aided translation. Provided here is a brief synopsis of each site and the relevant evidence as it pertains to this project.

3.1 Site Backgrounds

The first site in the sample is that of the ancient Romanesque Church of St. John Almedina (São João de Almedina), located in the city of Coimbra (Figure 3). Tenth-century Coimbra was described by the Arab chronicler Al-Razi as a strong city with a magnificent castle (Ferreira 2012). Ventura (1979) referred to it as: "a beautiful city which was situated on the river Mondego, who owned several fish species and their margin stood on the floodplain quite good for farming. In addition, it was a city with plenty of olive trees that produce delicious olive oil" (p. 55).

In the 13th century, Coimbra was one of the most important urban centers of Portugal, along with Lisbon and Santarém. However, Coimbra differed from the others due to its preference as a residential center for royalty (Gomes, 1998). Considered a mostly urban area, Coimbra was also represented by many rural areas and the city proper was divided into suburbs and Almedina. The

suburbs housed the merchants, artisans, and blacksmiths, while the area within the walls, the medina, was inhabited by the clergy, the canons' seat, and nobility (Alarcão, 2008; Cunha, 1994). The medina itself was considered an intramural space framed by the castle stronghold and the placement of small settlements around the church (Alarcão, 2008).

It is unknown when the church of St. John Almedina was founded but it was likely before the middle of the 11th century (Correia & Gonçalves, 1947 as cited in Ferreira, 2012). This Roman Catholic Church has been in use since at least the end of the 12th century and was formally used well into the late 15th or early 16th century. In the 17th century, Earl-Bishop John Melo rebuilt it as an Episcopal chapel and it was used as such until 1854 (Ferreira, 2012). Currently, St. John Almedina houses the National Museum Machado de Castro (Figure 4).

Figure 4. Church St. John Almedina. Photo courtesy of Dr. Francisco Curate.

In the mid-1940s, excavations were carried out in the church cemetery by archaeologist Dr. Bairrão Potter, and a portion of the human osteological analysis was performed by Maria Augusta Neto (Cunha, 1994). The area where the excavation occurred and the medieval human remains exhumed is currently within the courtyard of the National Museum Machado de Castro. Unfortunately, no archaeological report from these excavations has yet been located (Cunha, 1994).

It is possible that a few individuals from this site came from the Renaissance era; however, due to the lack of archaeological records and the end of its use as a Roman Catholic Church in the early 16th century, the parsimonious term "medieval" is used (Ferreira, 2012). While the status of the individuals exhumed from this site is unknown, its location within the medina makes it likely that the individuals interred here held positions from the clergy, canons' seat, and nobility.

The skeletal material was transported and housed at the facilities of Life Sciences at the University of Coimbra, where the material was inventoried and cleaned. Ferreira's (2012) thesis on the prevalence of osteoporosis among four medieval populations states that the skeletal material recovered from this site is well preserved and consists of a total of 40 individuals. A total of 24 individuals from this site are used in the dietary modeling portion of this study, and I was able to analyze 19 of these individuals in the association between diet and bone mineral density (BMD).

The second site is located from within the confines of the Convent of San Francisco (Convento de São Francisco) in the city of Santarém, what would become one of the most important cities in Portugal during the medieval era (Figure 3). In the middle of the second century the Romans settled here next to the river Tagus, naming the community *Scallabis* (Bastos et al., 2004 as cited in Ferreira, 2012). In the fifth century, after the fall of the Roman Empire, Visigoths inhabited Santarém until it was later invaded by Muslims. It was the setting for warfare between the Christians and Moors until 1147, when King Afonso Henriques made the conquest that added the region to the kingdom of Portugal (Ferreira, 2012). Santarém was chosen as the primary residence of many kings and nobles. Economic and political development allowed the construction of large churches, convents and other monuments between the twelfth and fifteenth centuries, granting the city the moniker of "the Gothic Capital" (Teixeira, 1994 as cited in Ferreira, 2012).

The Convent of San Francisco is a classic example of Gothic Mendicant architecture, and the ascetério (or monastery meditation room) was founded in 1242 by D. Sancho II with the establishment of the Franciscans in the city. However, its construction ended abruptly in the second half of the century during the reign of Fernando (Ferreira, 2012). With the extinction of religious orders in 1834, the convent suffered at the hands of military campaigns and a fire in 1940 solidified its state of degradation (Ferreira, 2012; Ramalho, 2001) (Figure 5).

In 1990, the archaeological excavations at the Convent of San Francisco uncovered a number of graves in both the nave of the church and the side chapels in the area of the churchyard. Skeletal material, burial context and fragments of tombstones provide some data regarding the people buried here (Ramalho, 1999). Analysis carried out by Maria Ramalho has contributed greatly

Figure 5. Convent of San Francisco in Santarém. Photo courtesy Dr. Francisco Curate.

to our understanding of the community interred there; significant also is the work of Drs. Eugenia Cunha, Claudia Umbelino, and Ana Maria Silva – these professionals undertook a multifocal analysis and studied the burials and types of graves, in addition to the identification and study of the early stages of construction of the convent (Ferreira, 2012).

Historic records and grave stones from the 15th century indicate that social inequalities continued to exist even beyond death. Lifeways, such as social status, are often recognized at the death of an individual so that social positions held in life followed them into the grave; thus, the burials of nobility and peasantry would be remarkably different in their appearances (Binford, 1971; Cohen & Bennett, 1993; Saxe & Gall, 1977; Schweich & Knüsel, 2003).

Grave site epitaphs reveal that individuals of nobility were mainly buried in places like the convent of San Francisco, as the nobility had the privilege and financial resources to be buried in these sacred places (Ramalho, 2001). However, Ramalho (2001) also notes the existence of epitaphs that list individuals of varying social classes: Notary, Master of Precious Stones, Treasurer of the Chamber of Santarém, Locksmith, and Confectioner, indicating that wealth sufficient for these "lower class" individuals to afford such a desirable burial location. In some ways, this may be analogous to the phrase, "keeping up with the Joneses," allowing individuals to exhibit higher social standing at the time of death so as to emulate the higher social classes, such as nobility (Binford,

1971). While it appears as if most buried at the convent were affluent members of society it is, however, unknown exactly what their social position may have been.

The partial excavation at the Convent of San Francisco recovered a total of 132 individuals. Of these individuals, 103 were adults of both sexes and different age groups (Silva et al., 1999). Transported to the Institute of Anthropology at the University of Coimbra, the skeletal material was inventoried according to the guidelines of the department (Ferreira, 2012). A total of 30 individuals from this site are utilized in the dietary modeling portion of this study, and 19 of these individuals were included in the study of the relationship between protein source and BMD.

The third and last site included in the sample is the Christian Necropolis of Cacela-a-Velha situated on the southern coast of Algarve (Figure 3). Historical references about the village and its fortress date back to Phoenician times, approximately 804 BC, and it is mentioned as being an important commercial and religious center within the region (Curate, 2004; Sánchez, 2000). During the second half of the 12th century, Cacela became an important defensive center designed to receive Muslim troops from the north of Africa, but in 1240 AD it was conquered by the current king of Portugal, D. Sancho II. Although it fell back into the hands of the Moors, it was regained by D. Paio Peres Correia, Master of Santiago, in 1242 AD in the name of the Kingdom of Portugal (Lopes, 1841 as cited in Ferreira, 2012; Sánchez 2000).

Following the defeat of the Islamic population at Cacela, a Christian church with contiguous necropolis was built. It is estimated that this burial site was most active for the next two centuries, and radiocarbon dates from the human skeletal remains indicates the samples to have been interred from the beginning of the 13th century through the early 14th century (Garcia & Curate, 2004) (Figure 6). Historical records indicate that the necropolis still existed in 1565 AD and was active until the end of the century when it was finally abandoned (Curate, 2004).

Archaeological excavations took place in 1998 and 2001 at the site identified as 'Floodplain in Cacela Velha' (Figure 7). Under the guidance of Dr. Francisco Curate, 74 individuals were exhumed from the necropolis (Curate 2004). Other than east-west orientation of Christian burials, no discernible patterns of inhumation could be assessed. The only grave goods collected were pins used to hold linen/wool shrouds in place; this lack of funerary goods and the modest burial structures reflect the lower social class and economic status of a region that lived under times of instability and conflict (Garcia & Curate, 2004).

Buried in sand, the well-preserved remains represent single as well as secondary interments (Figure 8). Transported to the Department of Life Sciences at the University of Coimbra, the

Figure 6. Christian church at Cacela Velha. Photo courtesy of Dr. Francisco Curate.

Figure 7. Excavation at the Christian necropolis of Cacela. Photo courtesy of Dr. Francisco Curate.

skeletal material was analyzed and labeled according to guidelines of the department; some remains were coated in thin layers of varnish for preservation (Curate, 2004). A total of 34 individuals from this site are utilized in the dietary modeling portion of this study, and 25 of these individuals were included in the study of the relationship between protein source and BMD.

These three sites – St. John Almedina, the Convent of San Francisco, and Cacela-a-Velha – have divergent histories that reflect varying lifestyles and traditions. The context of each site also

Figure 8. Individuals exhumed from the site of Cacela.
Photo courtesy of Dr. Francisco Curate.

influences their interpretation as it relates to differential dietary and pathological results. Lifeways are often ascribed to individuals at their death so that mortuary patterns closely reflect their socioeconomic status and social persona once held in life, and consequently also likely reflect subsistence patterns (such as, utilization of different dietary protein source) (Binford, 1971; Cohen & Bennett, 1993; Saxe & Gall, 1977; Schweich & Knüsel, 2003). The lack of data at the level of the individual burial and absence of records from each site leave many questions about the status of the individuals buried there. Nevertheless, when coupled with what we know about medieval dietary trends, the data will allow for some interpretation of the inter- and intra-regional dietary patterns.

3.2 Medieval Dietary Patterns

Our knowledge of medieval diet in Portugal largely relies upon historic documents. Previous stable isotope analyses have investigated diet within the same region but not the same time frame, while other studies were based on the same time frame but not the same region (Alexander, Gerrard, Gutiérrez, & Millard, 2015; Lubell & Jackes, 1994; Waterman, 2012; Waterman, Silva, & Tykot, 2014). These resources can still be used as a comparison to the results of the present study and will be discussed in detail in Chapter 4.

On a regional scale, information about medieval Portuguese diet comes from market regulations, amended constitutions, and court chronicles. Records of municipal law and pageants

and festivities offer insight into what was being consumed, in addition to the social distinctions of what was allowed to be traded/sold and how it was done. Texts from Christian, Muslim, and Jewish sources provide insight into temporal changes in diet as medieval Portugal underwent changes of authority (Constable, 2012; Livermore, 1973; Marques, 1971; O'Callaghan, 1975).

Even though the individuals examined in this study are Christian, many Muslims lived in the same areas, trading the same wares (Constable, 2012). Records from Muslim market regulations from the twelfth century reveal a wide range of dietary protein sources that were likely consumed by people outside of the Muslim religion. Muslim records state that rabbits and poultry were not allowed to be sold around the mosque, eggs were sold along with cheeses, and meat and fish were sold by weights (Constable, 2012). The only animal whose slaughter is discussed is sheep; however, they do state that any animal good for field work should be spared and only if they have a defect can they be slaughtered (Constable, 2012). Fish was sold fresh and salted, and was decreed to "not be washed in water for this makes it go bad." (Constable, 2012, p. 229). In addition to animal products, bread, flour, and even truffles are mentioned as market wares. However, eating truffles was considered a sign of degeneracy and self-indulgence, so they were not to be sold around the mosque (Constable, 2012).

Charters during the thirteenth century reveal laws placed to protect the Muslim community during the conquest of eastern Islamic Spain. In addition to inheritance, religious rights, and taxes, these laws stipulate their security to raise livestock, sow foodstuff, keep orchards, and maintain beehives (Constable, 2012). They illustrate the variability of foodstuffs available to people then: wheat, barley, millet, panic grass, flax, onions, cucumbers, fruit trees, and vineyards. Animal protein is simply referred to as "livestock" or "domestic animals" (Constable, 2012).

An account of the preparation for a royal wedding in the 15th century illustrates the grandeur involved in noble pageantry. Feasts lasted days, guests were offered wine from silver fountains, and ponds were stocked with trout and barbels (freshwater fish). As fish were caught they were presented to the bride to be feasted upon later. Additionally, wooded areas were stocked with bear, boars, and deer, and although it is not explicit that they were part of the feast it is likely that many of these animals made it to the table to be consumed (Constable, 2012).

Marques (1971) states we have few sources regarding what food was consumed in Portugal during the Middle Ages; the first known book of culinary recipes dates to the fifteenth century. However, Marques (1971) also presents information gathered from a multitude of sources and provides a collection of recipes along with dietary social norms (e.g., meal times).

The medieval Portuguese diet was mainly based on cereals, meat, fish, and wine. Greens and vegetables were not commonly consumed by the upper class but were consumed in great quantities by the lower class. The cabbage family as well as beans, broad beans, broccoli, lettuce, cucumbers, radishes, mushrooms, carrots, turnips, asparagus and other garden vegetables were all eaten as well (Marques, 1971). Important grains included wheat, barley, and millet. When grains were scarce, legumes such as broad beans, peas, lentils and chickpeas were used as substitutes for bread. After the 14th century, grain was often scarce in the kingdom of Portugal and broad beans were imported to meet the needs of the people (Marques, 1971). However, the interior regions, such as Beira, did not resort to the broad bean and instead ate chestnuts in place of bread (Marques, 1971).

> Marques (1971) suggests another staple of the diet was mainly meat:
> There were meats from the slaughterhouse – beef, pork, mutton, goat. In Coimbra in the twelfth century, the best prices were quoted for pork and fat mutton, followed by beef and goat; Evora in 1280, as in 1384, the price of beef was twice that of pork and more than twice that of mutton and goat. (p. 17)

The high cost of beef suggests that only the wealthy could have afforded it. However, game and poultry were consumed by both upper and lower classes of people. Hunting was a pastime for nobility and presented an important source of subsistence for the peasantry. Market place prices were listed for meat of the deer, zebro (now extinct cattle), roebuck, hare, and bear; fowl consisted of partridge, crane, wild duck, heron, calander lark, and the great bustard (Marques, 1971). Poultry consisted of familiar birds such as chickens, ducks, geese, pigeons, pheasant, peacock, and doves (Marques, 1971). Additionally, dairy products and eggs were eaten in large quantities – practically all recipes listed by Marques (1971) called for eggs.

Fish formed a staple of the medieval Portuguese diet but was particularly consumed by the lower classes, and the consumption by nobility and clergy stemmed from their religious edicts (Marques, 1971). Dietary restrictions placed by the church would have influence on the consumption of fish, particularly on Fridays. Meat was prohibited for Catholics approximately 68 days a year, and there could be no meat, eggs, cheese, butter, or even oily fish consumed on those days (Marques, 1971). Instead, vegetables, fruit, and small fish were recommended by the church (Marques, 1971).

> One of the fish most frequently eaten by the Portuguese in the middle ages seems to have been whiting (*peixota*), found in almost all the documentation that specifies varieties of fish. Sardines, conger, eels, shad, surmullet, and lamprey eels were also frequently seen on the tables of every social class. Red mullet, snapper or porgy, tuna, tuna, trout, flounder, *bizugos*, shark, turbot, sea bream, and many other fish were objects of culinary art. Meat from the whale and the porpoise was also eaten. Shellfish (like tellin shell and mussels) and crustaceans (like lobsters and crabs) were common. (Marques, 1971, p. 21)

It is obvious that areas of Portugal enjoyed a variety of fresh fish; however, a great deal of dried, salted, and smoked fish was also eaten and made its way to the interior. With a lack of refrigeration and warm climate, drying fish enabled its exportation over great distances and long periods of time. At the end of the 14th century sardines were smoked for export from Lisbon to Seville and/or Aragon (Marques, 1971).

In summary, historical sources suggest that medieval Portuguese dietary regimes incorporated wide varieties of protein that varied with social standing and religious status. These included protein groups that should have isotopically distinctive signatures: terrestrial animal protein (meat, dairy, eggs), marine fish, C3 resources (C3 plants such as wheat and/or animals that ate those plants) and C4 resources (millet and/or animals that ate millet). Other isotopically distinctive protein sources included legumes and freshwater fish.

3.3 Summary

As previously stated, the individuals examined here come from three different regions. The historic records indicate that each burial site may represent different social classes. Historic records, indicate that the burials recovered from the Convent of San Francisco represent individuals that could afford to be buried there – people of wealth and nobility (Ramalho, 2001). Based on what we know from historic sources on medieval dietary patterns, the wealthy individuals at the Convent of San Francisco likely ate mainly terrestrial protein sources supplemented with fish due to religious precepts (Marques, 1971).

Although little information exists on the social standing of individuals buried at the site of St. John Almedina, historical records indicate that the area within the walls where the church is located (the medina) was inhabited by clergy, the canons seat, and nobility (Alarcão, 2008; Cunha,

1994). It stands to reason then that the individuals buried in the church cemetery, located within the medina, held an affluent status. Again, and as at the site of the Convent of San Francisco, medieval dietary trends tell us that it is likely these individuals would have consumed mainly terrestrial protein sources supplemented with fish (Marques, 1971).

Due to its military and commercial background and its location on the coast, the site of Cacela-a-Velha is where the most diverse classes of society may be represented (Curate, 2004; Sánchez, 2000). If this is true, the diets here should incorporate a broad range of foodstuffs. Given this, along with its location on the coast, both terrestrial and marine dietary protein would likely be consumed.

The following chapter covers the methods and materials used within this study, including those performed by the bioarchaeologists at the University of Coimbra. The chapter is divided into sections that first cover general assessment of the skeletal remains in addition to the methods used in obtaining bone mineral density and the diagnosis of osteoporotic individuals. Additional sections discuss the methods and statistical analyses used to assess the relationship between protein source and bone mineral density. The chapter concludes with a discussion on the methods of diet modeling.

Chapter 4 MATERIALS AND METHODS

In this chapter I will discuss how bioarchaeologists analyzed the above-referenced samples in Portugal, as well as the methods used for the current study. As discussed, the human skeletal samples come from three separate archaeological sites in Portugal. The necropolis of Cacela-a-Velha (n = 34), the central site of the Convento São Francisco in Santarém (n = 30), and the churchyard site of São João de Almedina in Coimbra (n = 24). All individuals were interred during the middle to late medieval periods, with the possibility of a few individuals from São João de Almedina deriving from the early Renaissance era (13^{th} – 16^{th} century).

Taking into account differences in male and female physiology and thus differences in their response to disease, males and females are considered separately (Coale, 1991; DeWitte, 2010; Grauer & Stuart-Macadam, 1998; Hill & Upchurch, 1995). When exploring the relationship between health conditions such as osteoporosis (OP) and diet, it is particularly important to consider the sexes separately due to evidence that suggests males and females reach peak bone mass at different rates, as well as the occurrence of menopause in women. Additionally, it is likely that males and females within these societies exhibited differences in diet which further precludes combining the sexes (Coale, 1991; DeWitte, 2010; Grauer & Stuart-Macadam, 1998; Hill & Upchurch, 1995).

Each of the three sites is also considered individually because their discrete geographical locations may result in different stable isotope values due to varying isoscapes. Statistical analyses (discussed below) substantiate the consideration of each site separately.

4.1 Previous Studies

In order for patterns of bone loss to be meaningfully interpreted, the age and sex of the individuals need to be accurately determined. However, assigning age/sex to archaeological skeletons can be difficult due to a range of complicating factors. The inherent challenges of age and sex identification, when combined with ambiguity in relation to the identification of OP, can limit the amount of information gathered about past skeletal health.

Additionally, the analysis of the relationship between protein source and bone mineral density (BMD) was restricted to those who could be assessed for BMD and subsequently OP. Therefore, the femora could exhibit no taphonomic changes that would interfere with evaluation of BMD (Curate, personal communication, August 12, 2014). This resulted in a sample of 63 individuals for that portion of the study. The dietary modeling portion of the study included a total

of 91 individual skeletal remains, randomly selected based on the ability to identify sex and age at the time of death.

4.1.1 Sex Assessment

The individuals included in this investigation were assessed for sex and age at death by bioarchaeologists at the Department of Life Sciences, University of Coimbra, Portugal (Ferreira, 2012). The methods used to diagnose sex included both morphological traits and metric data; the combination of which improved the accuracy in identification (Ferreira, 2012). Two morphological methods were used to identify sex from individual os coxae: the Ferembauch, Schwidetzky, and Stloukal (1980) and the Bruzek (2002) methods, the second having been developed based on Portuguese materials (Ferreira, 2012).

Where possible, the osteometric method by Murail, Bruzek, Hoüet, & Cunha (2005) was also employed. The skull was assigned a sex using the morphological method described by Ferembauch et al. (1980) and with the use of comparative illustrations (Buikstra & Ubelaker, 1994). The long bones (mainly femur, humerus, and radius) were also used to determine sex, based on methodologies developed by Wasterlain (2000). Lastly, discriminant functions developed by Silva (1995) to determine the sex of individuals from their talus and calcaneus were used. All metric data were obtained with the use of standard and digital calipers (Ferreira, 2012). This process resulted in the identification of 51 males and 37 females.

4.1.2 Estimation of Age at Death

The individuals analyzed in this study were placed into one of three age groups: group one consists of individuals aged 20-29 years (adult), group two of individuals aged 30-49 years (middle-aged adult), and group three individuals aged 50+ years (elderly). The estimation of age at death was determined based on the observation of the degree of synostosis of the cranial sutures, degenerative changes of the sternal ends of the ribs, and changes to the pubic symphysis and auricular surfaces of the os coxae (Bass, 2005; Işcan, Loth, & Wright, 1985). The pubic symphysis was analyzed according to the Brooks and Suchey method (1990). Changes to the auricular surface were also observed when possible (Ferreira, 2012). This analysis resulted in the identification of 16 individuals in the 20-29 year age range, 37 between 30 and 49, and 35 older than 50.

4.1.3 Identification of Osteoporosis

The most commonly used methods to study bone density in past populations are dual X-ray absorptiometry (DXA) and radiography (Mays, 2008b). Diagenetic changes may distort bone density readings; however, these techniques have been extremely useful in identifying broad trends of bone loss and assessing risk of osteoporosis (Brickley & Agarwal, 2003; Curate, 2005, 2014a; Curate et al., 2013).

The individuals included in this study were assessed for OP by bioarchaeologists at the University of Coimbra, Portugal through the use of DXA. Modern clinical studies have documented the usefulness of this technique for predicting fracture risk and it is the current standard in bone mass assessment methodology (Bonnick, 2010; Lenchik et al., 2004). Importantly, this method also provides an accurate diagnosis of OP in skeletal samples from archaeological contexts (Agarwal, 2008; Mays, 2008a).

Dual X-ray absorptiometry allows for the measurement of bone mineral content and bone mineral density (BMD) of practically any bone in the skeleton. It provides a measure of bone mineral density as the amount of hydroxyapatite in grams per square centimeter of bone (g/cm^2) (Curate, 2014a; Lenchik et al., 2004). Note that, DXA does not measure bone volumetric density, nor is it entirely standardized for bone size. Despite these limitations, DXA is considered the clinical standard for the measurement of BMD (Bonnick, 2010; Lenchik et al., 2004). To diagnose OP, raw measures of BMD are compared to reference values for healthy young adults.

Following standards set forth by the World Health Organization (WHO), the determination of OP in each individual is made according to their T-score, a standardized score relating to bone densitometry. T-scores are calculated by subtracting the mean BMD of a healthy reference population from the individual's measured BMD and dividing by the standard deviation of the same reference population. The WHO definition of OP for post-menopausal Caucasian women is a T-score less than or equal to -2.5. Their criteria for the determination of osteopenia (the precursor to OP) is a T-score between -1.0 and -2.5, while normal is considered equal to or above -1.0 (Hamdy, Petak, Lenchik, 2002; Kanis, 1994). However, these criteria are also commonly applied to men and premenopausal women, including non-Caucasian individuals, which may be problematic because BMD can vary between sexes and among ethnic groups (Binkley, Schmeer, Wasnich, & Lenchik, 2002; Hui et al., 1999; Yu et al., 1999). Nevertheless, the clinical standard is to apply the same WHO diagnostic criteria to men as they do to women, and the International Society for Clinical Densitometry recommends using Caucasian reference data for non-Caucasians (Binkley et al., 2002).

Typically, two skeletal sites are measured by DXA, the posterior-anterior lumbar spine and the proximal femur. Both are measured because clinical studies indicate that approximately 20-30% of patients have significant spine/hip discordance in which the T-scores of one site are of a different diagnostic category than the other site (Varney, Parker, Vincelette, & Greenspan, 1999; Woodson, 2000). In patients with acute degenerative disease of the spine, hip BMD offers more accurate monitoring (Lenchik et al., 2004). Additionally, a forearm scan may be obtained in patients in which the spine and/or the proximal femur scans are invalid; fractures, implants, and severe degenerative disease may impede accurate diagnoses (Hamdy et al., 2002).

While studies of the lumbar spine and hip region are the most common in clinical studies, bioarchaeological studies focus on the proximal femur (Brickley & Agarwal, 2003; Lees, Molleson, Arnett, & Stevenson, 1993; Lenchik et al., 2004; Mays, Lees, & Stevenson, 1998; Mays, Turner-Walker, & Syversen, 2006). This is largely due to better preservation of the femur in archaeological contexts. The radius has also been used to assess bone loss in human skeletal remains but this can be problematic because the DXA scanner may be unable to detect bone mass in the regions of interest (Curate, 2005; Ferreira, Albuquerque, Ferreira, Cunha, & Curate, 2012; Zaki, Hussien, & El Banna, 2009).

The individuals included in this study were scanned with a Hologic QDR 4500C Elite, located in the Department of Nuclear Medicine Center and Hospital at the University of Coimbra, Portugal. The scanner was calibrated daily using "phantoms" supplied by the manufacturer (Curate, 2010 as cited in Ferreira, 2012; Silva, Carapito, & Reis, 1999). Left femora were chosen for this analysis and only when this element was missing or gross diagenetic changes had occurred were the right femora utilized (Ferreira, 2012). Each bone was placed in a low-density cardboard box and covered with rice as a substitute for soft tissue (Brickley & Agarwal 2003; Curate, 2010; Ferreira, 2012; Mays et al., 2006; McEwan, Mays, & Blake, 2004). The femora were placed (singly) in an anterior-posterior position, with the diaphysis parallel to the central axis of the scanner (Figure 9).

The proximal femur was scanned in five specific regions of interest (ROI): femoral neck, Ward's Area, trochanter, intertrochanteric region, and "total hip" (i.e., the sum of the neck, trochanter, and intertrochanteric areas) (Ferreira, 2012) (Figure 10). Each of these regions comprises different percentages of trabecular and cortical bone (Bonnick, 2010). In clinical contexts and in archaeological studies the femoral neck is used most often for the diagnosis of osteoporosis; however, use of the lowest T-score of the total hip, neck, or trochanter is also recommended (Curate, 2010; Ferreira, 2012; Lenchik et al., 2004).

Figure 9. Hologic QDR 4500C Elite, located in the Department of Nuclear Medicine Center and Hospital at the University of Coimbra, Portugal. Photo courtesy of Ferreira 2012.

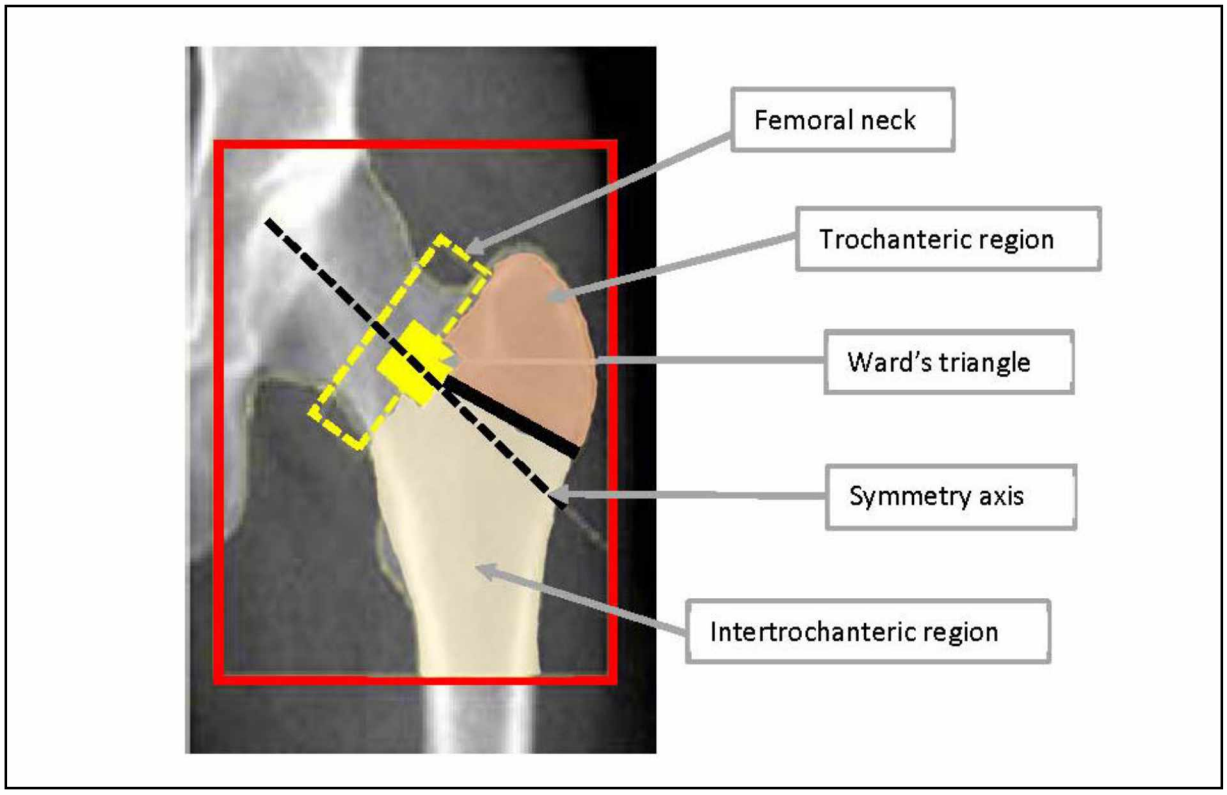

Figure 10. Illustration of the ROI for the analysis of bone loss by DXA in the proximal femur. Image modified from National Institute of Health image of hip scan.

4.2 This Study

4.2.1 Determination of Osteoporotic Individuals

Although skeletal material from 91 individuals was made available for the present study, only 79 were analyzed for BMD by the bioarchaeologists at the University of Coimbra. Out of these 79 individuals, only 63 had bone density data on the femoral neck region. For this study the femoral neck T-score was used to diagnose osteoporotic development (Ferreira et al., 2012; Lenchik et al., 2004). Using the T-scores provided by Dr. Curate at the University of Coimbra, Portugal, I followed WHO criteria for the determination of osteopenia and OP. T-scores below or equal to -2.5 were considered osteoporotic, T-scores between -1.0 and -2.5 were considered osteopenic, and T-scores equal to or above -1.0 were considered normal (Lenchik et al., 2004) (Table 1).

Table 1. Total number of individuals analyzed for osteoporosis by site.

SITE	DIAGNOSIS	# of Male Individuals	# of Female Individuals	TOTAL
Almedina (n = 19)	Normal	9	6	15
	Osteopenic	0	3	3
	Osteoporotic	0	1	1
Cacela (n = 25)	Normal	17	8	25
	Osteopenic	0	0	0
	Osteoporotic	0	0	0
S. Francisco (n = 19)	Normal	5	6	11
	Osteopenic	2	4	6
	Osteoporotic	1	1	2
TOTAL		34	29	63

4.2.2 Sample Collection and Preparation for Isotopic Analysis

Subsamples of bone were taken from the elements provided to me by Dr. Curate. These consisted mainly of ribs (n = 79) in addition to a few phalanges (n = 4), long bones (radii n = 3, fibulae n = 3), and a single ilium fragment. The portion of bone sampled for isotopic analysis was chosen with a goal of reducing the chance and/or amount of contamination. This portion mainly consisted of diaphyseal segments of bone; center portions were chosen with the idea that

distal/proximal ends of bone are more likely to uptake environmental contaminants (e.g., humic acids), increasing the chance of distorted stable isotope results. Healthy bone that was macroscopically determined to be free of pathogenesis was chosen to avoid the stable isotope variation found in pathological bone (Katzenberg & Lovell, 1999; Olsen et al., 2014).

These sections of bone were sanded under a fume hood using a Dremel rotary hand tool fine grain drum sander to remove any surface contamination, including varnish, which was apparent on a few samples. Although the exact compound used to coat the bone is unknown, it was treated as any other surface contaminate and removed completely. Additionally, studies in the treatment of consolidants on bone prepared for stable isotope analysis have shown that preservative materials do not disqualify them for chemical analysis, and often the consolidants can be easily removed (e.g., sanding) (Moore, Murray, & Schoeninger, 1989).

In the case of phalanx samples, the entire bone was sanded and utilized in the analysis; otherwise, a section of bone was excised using a Dremel rotary hand tool diamond wheel. A sharp blast of air was utilized to rid the specimen of bone dust before it was placed into its own specimen bag. The excised pieces of bone were then powdered with the aid of a mixer mill (Retsch MM200). Each sample was milled for 30 seconds at a frequency of 20 Hz, with a few samples having to be milled twice to achieve a grain size smaller than 0.3 mm. Once powdered, the samples were placed into individual glass vials and stored for collagen extraction.

The extraction of collagen from the powdered bone samples followed a modified Longin (1971) method. Approximately 0.04 grams of bone powder from each sample was weighed into a pre-weighed 1.5 milliliter microcentrifuge tube. The bone powder was demineralized in 0.5 M HCl (30 minutes x 3), rinsed, and soaked in 0.1 M NaOH (10 minutes x 2) to remove humic contaminants. Following another rinse cycle, the samples were then gelatinized in 0.001 M HCl with heating on a Thermomixer set to 70°C for approximately 24 hours. After centrifuging at 10,000 rpm for 10 minutes, the sample supernatant (containing solubilized collagen) was collected by a transfer pipette and placed into a clean, pre-weighed 1.5 milliliter microcentrifuge tube. Samples were then frozen and placed on a freeze dryer for 48 hours. Weights of dried bone collagen were then obtained to estimate the collagen yield (%coll).

Approximately 0.3 mg of each collagen sample were weighed in a tin boat and analyzed on the Thermo Finnigan Delta-Plus XP continuous flow isotope ratio mass spectrometer, coupled to a Costech Elemental Analyzer (ECS 4010), at the Alaska Stable Isotope Facility (ASIF), housed on the University of Alaska Fairbanks campus. Instrument precision for this lab is <0.2‰ based on

repeated analysis of an in-house standard (animal peptone) (Tim Howe, ASIF Manager, personal communication, September 15, 2013). Carbon and nitrogen stable isotope reports also include sample %N and %C. Samples were assessed for collagen quality using standards described by Ambrose (1993) and van Klinken et al. (2000).

Faunal remains from the sites were not available and consequently not directly sampled for this study. Instead, a faunal baseline was estimated using previously published samples. These samples consist of cow, pig, sheep/goat, chicken, and marine fish from reasonably similar regions of the Iberian Peninsula. These faunal samples span the period of 2800 BC-16th century (Alexander et al., 2015; Fuller, Márquez-Grant, & Richards, 2010; Waterman et al., 2011).

4.2.3 Statistical Analyses

All statistical analyses were performed and calculated with IBM SPSS Statistics 21 software and Microsoft Office Excel. Initial examination of the data included exploring assumptions for parametric statistical tests and identifying outliers for the variables $\delta^{13}C$, $\delta^{15}N$, and BMD by subgroup (males and females at each of the three sites). The assumption of normally distributed data was assessed via visual analysis of data plots and through the use of the Kolmogorov-Smirnov test of normality, while the assumption of homogeneity of variance was examined using Levene's test (Norušis, 2012). Outliers were determined by calculating Z-scores and Cook's distance; any case with an absolute value of 2.5 or greater (i.e., 2.5 standard deviations above/below the mean) was designated an outlier and removed from the dataset (Norušis, 2012). Three individuals (AL027, CA037, and SF137) were deemed outliers for the variables $\delta^{15}N$, $\delta^{13}C$, and $\delta^{13}C$ (respectively) and were consequently removed from the analysis.

Data inspection began with the assessment of normality for each data set – i.e. those samples utilized in the analysis of the relationship between protein source and BMD (n = 63), and the larger sample used to model diet at each site (n = 88). The variables of interest were assessed through visual inspection of distributions and statistical normality tests (Kolmogorov-Smirnov) separately by each site and by sex. The results of the normality tests for both data sets can be viewed in Table 2 and Table 3.

The results of the bone mineral density (BMD) data set show that males and females are normally distributed for BMD in all three sites. When analyzing the variable $\delta^{13}C‰$, two out of the three sites are normally distributed; however, the Convent of SF exhibits non-normally distributed data in the female subgroup. Here, female $\delta^{13}C‰$ values are slightly higher than the rest of the

Table 2. Results of the Kolmogorov-Smirnov normality tests for BMD and dietary values ($\delta^{13}C$ and $\delta^{15}N$) data set.

	BMD				$\delta^{13}C$				$\delta^{15}N$			
	Male		Female		Male		Female		Male		Female	
Site	df	*p*	df	*p*	df	*p*	df	*p*	df	*p*	df	*p*
SJ de Almedina	9	0.200	10	0.200	9	0.200	10	0.200	9	0.042	10	0.200
Convent of SF	8	0.200	11	0.200	8	0.085	11	0.037	8	0.200	11	0.200
Cacela	17	0.123	8	0.200	17	0.200	8	0.200	17	0.200	8	0.200

Table 3. Results of the Kolmogorov-Smirnov normality tests for the dietary modeling data set.

	$\delta^{13}C$				$\delta^{15}N$			
	Male		Female		Male		Female	
Site	df	*p*	df	*p*	df	*p*	df	*p*
SJ de Almedina	13	0.200	11	0.200	13	0.008	11	0.200
Convent of SF	13	0.041	17	0.098	13	0.200	17	0.200
Cacela	25	0.200	9	0.200	25	0.200	9	0.200

sample and slightly skew the results. For the variable $\delta^{15}N‰$, only the site of S. J. de Almedina illustrates' a non-normal distribution – two males exhibit $\delta^{15}N$ values that are lower than the observed mean (Table 2).

The results of the dietary modeling data set show that males and females are normally distributed at two of the three sites for $\delta^{13}C‰$; males at the Convent of SF exhibit non-normally distributed data due to two outliers exhibiting high $\delta^{13}C‰$ values even after removing previously identified outliers. For the variable $\delta^{15}N‰$, the site of SJ de Almedina exhibits a non-normal distribution in males only; three individuals were outliers for $\delta^{15}N‰$ exhibiting values lower than the observed mean (Table 3). Additionally, these individuals were identified after having removed extreme outliers; and while statistical tests indicated some departure from normality, visual inspection of the data plots indicated that the differences were not severe, and so parametric tests were used.

Additionally, Levene's test for equality of variances provided significance levels that were consistent with treating group variances as equal in two out of the three sites, ensuring that the

assumption of homogeneity of variance had not been violated. Homogeneity of variance was not found at the site of SJ de Almedina for the variable BMD within the BMD data set, and Levene's test for equality of variances provided significance levels that were consistent with treating group variances as equal in all three sites for the Diet Modeling data set (Table 4 and Table 5).

Table 4. Levene's test of equality of variances between males and females for the BMD data set.

	BMD		$\delta^{13}C$		$\delta^{15}N$	
Site	F	*p*	F	*p*	F	*p*
SJ de Almedina	5.612	0.03	1.187	0.291	0.13	0.723
Convent of SF	0	0.991	2.17	0.158	0.351	0.561
Cacela	0.036	0.851	3.037	0.095	0.749	0.396

Table 5. Levene's test of equality of variances between males and females for the diet modeling data set.

	$\delta^{13}C$		$\delta^{15}N$	
Site	F	*p*	F	*p*
SJ de Almedina	1.187	0.291	0.13	0.723
Convent of SF	1.573	0.22	0.024	0.878
Cacela	1.45	0.237	0.33	0.57

The independent samples T test was used to test for mean differences in $\delta^{13}C$, $\delta^{15}N$, and BMD between sexes (separately by site), while One-Way ANOVA was used to test for mean differences among sites (separately for males and females). Both of these statistical analyses test the null hypothesis that there is no mean difference between groups. The results for both data sets are presented in the Chapter 5.

Simple linear regression was used to explore the relationship between BMD and protein source (as reflected in $\delta^{13}C$ and $\delta^{15}N$). Analyses were conducted separately for each of the six subgroups: males at the site of SJ de Almedina, females at the site of SJ Almedina, males at the site of the Convent of SF, females at the site of the Convent of SF, males at the site of Cacela, and

females at the site of Cacela. Linear regression analysis describes the relationship between a dependent variable, *x*, and an independent variable, *y*, in the form of an equation in which *y* is a linear function of *x*, as follows: $y=a+bx$. In this equation, a is the *y*-intercept and b is the slope, which describes the change in *y* for a one-unit increase in *x*. The statistical null hypothesis being tested for in these analyses is that there is no linear relationship between BMD and the dietary variable of interest ($\delta^{13}C$ and $\delta^{15}N$). For all statistical tests, the null hypothesis was rejected if the p-value was less than or equal to the significance level (α) of 0.05.

4.2.4 Diet Modeling Analyses

Diet modeling was conducted separately from the analysis of the relationship between diet ($\delta^{13}C$ and $\delta^{15}N$) and BMD. Since BMD data was not necessary for dietary modeling, an additional 25 individuals were incorporated into this portion of the study. The carbon and nitrogen isotope ratios from this study were compared to other data from the Iberian Peninsula in order to provide additional context for the study of the relationship between protein source and BMD (Alexander et al., 2015; Fuller et al., 2010; Lubell & Jackes, 1994; Waterman et al., 2011).

By comparing mean stable isotope ratios from these other studies to the sites analyzed here, visual examination by scatter plot can indicate whether or not the values fall within typical ranges for the region. This ensured that the stable isotope ratios used in this study were supported and could be used with confidence. These mean values are presented in Tables 10 and 11 and Figures 18 and 19 in Chapter 5.

Finally, I performed a simple visual analysis of isotope ratio plots. Because of the possibility for medieval Portuguese individuals to have consumed both marine foods and millet (a C4 plant), both carbon and nitrogen stable isotope ratios were used to interpret sources of dietary protein. The results will be discussed in the Chapter 5.

Chapter 5 RESULTS

This chapter describes the results obtained from descriptive analysis and statistical tests. First presented is the data on the analysis of the relationship between stable isotope ratios and BMD, followed by a presentation of the results of the dietary modeling. Sites were kept discrete, and males and females were kept separate, unless otherwise noted. In this section, *p*-values are considered statistically significant if less than or equal to 0.05. Discussion of these results can be found in the Chapter 6.

5.1 Sample Quality

Carbon and nitrogen stable isotope results are shown in Table 6, Table 7, and Table 8 (by site). As stated by Ambrose (1993), collagen preservation can vary greatly within sites, so it is important to determine bone collagen concentrations and present this data for others' independent evaluations. Quality indicators for collagen preservation include %N > 5% and %C > 13%, atomic C:N ratios between 2.9 and 3.6, and percent collagen yields greater than 1% (Ambrose, 1990; DeNiro, 1985; van Klinken et al., 2000). Percent collagen yield above this threshold is considered to hold its *in vivo* integrity. All collagen samples fell within the acceptable limits.

5.2 Stable Isotope Results

Table 9, Table 10, and Table 11 show summary statistics for $\delta^{13}C$ and $\delta^{15}N$ at each site for the BMD data set alone. The $\delta^{15}N$ values from the site of São João de Almedina (SJ de Almedina) range from 10.3 to 12.1‰ (mean = 10.99‰) in females and 8.9 to 11.7‰ (mean = 10.6‰) in males. The $\delta^{13}C$ values from this site range from -18.8 to -14.5‰ (mean = -16.6‰) in females and -18.1 to -15.4‰ (mean = -16.8‰) in males. The $\delta^{15}N$ values at the Convento de São Francisco (Convent of SF) range from 11.5 to 13.7‰ (mean = 12.7‰) in females and 11.9 to 14.5‰ (mean = 13.2‰) in males. The $\delta^{13}C$ values from S. F. range from -18.7 to -15.7‰ (mean = -17.7‰) in females and -18.3 to -16.4‰ (mean = -17.7‰) in males. At the site of Cacela-a-Velha (Cacela), the $\delta^{15}N$ values range from 9.4 to 11.7 (mean = 10.7‰) in females and 8.6 to 13.1‰ (mean = 11.3‰) in males. The $\delta^{13}C$ values from this same site range from -18.7 to -17.4 ‰ (mean = -18.0‰) in females and -18.9 to -15.7‰ (mean = -17.7‰) in males.

Table 6. Values expressing collagen quality indicators, stable isotope ratios, and element sampled at São João de Almedina.

Sex	Total		ID Number	Element Sampled	Conc C (%)	Conc N (%)	$\delta^{13}C‰$	$\delta^{15}N‰$	C:N Ratio	% Collagen Yield
Female			AL001	Rib	51.04	18.16	-16.90	10.82	3.28	4.76
			AL004	Radius	51.26	17.93	-16.87	10.33	3.34	2.58
			AL005	Radius	51.50	18.46	-16.19	11.20	3.26	5.19
			AL006	Rib	49.87	17.84	-15.15	10.64	3.26	3.30
			AL011	Rib	53.68	19.27	-15.84	10.33	3.25	4.55
			AL012	Rib	49.78	17.78	-14.46	10.33	3.27	3.22
			AL015	Rib	52.96	18.96	-17.54	11.44	3.26	8.90
			AL018	Rib	50.64	17.88	-17.23	10.94	3.30	3.27
			AL028	Fibula	53.11	18.97	-16.66	12.12	3.27	5.79
			AL029	Ilium	52.91	18.81	-18.83	10.71	3.28	5.84
			AL031	Radius	53.32	19.16	-16.77	11.96	3.25	14.62
	Total	N	11	11	11	11	11	11	11	11
		Mean			51.82	18.48	-16.58	10.98	3.27	5.64
		Std. Deviation			1.42	0.58	1.18	0.63	0.03	3.46
		Std. Error of Mean			0.43	0.17	0.36	0.19	0.01	1.04
Male			AL002	Rib	51.85	18.50	-18.07	11.00	3.27	3.97
			AL003	Rib	53.52	18.96	-17.71	11.65	3.30	7.64
			AL007	Rib	49.69	17.78	-16.45	11.10	3.26	0.89
			AL008	Rib	48.78	17.45	-17.67	9.36	3.26	2.97
			AL009	Rib	49.48	17.49	-16.42	10.99	3.30	2.00
			AL013	Rib	50.18	18.09	-15.80	10.87	3.24	4.20
			AL014	Rib	52.48	18.84	-17.66	9.66	3.25	5.71
			AL017	Int. Phalanx	52.22	18.64	-17.01	10.73	3.27	7.80
			AL019	Rib	50.09	17.99	-17.40	8.92	3.25	3.65
			AL021	Rib	51.56	18.35	-17.99	10.81	3.28	3.13
			AL023	Rib	48.53	17.35	-16.60	10.75	3.26	1.97
			AL024	Rib	51.28	18.35	-17.19	10.51	3.26	4.10
			AL030	Fibula	51.22	18.25	-15.35	10.97	3.28	1.20
	Total	N	13	13	13	13	13	13	13	13
		Mean			50.84	18.16	-17.02	10.56	3.27	3.79
		Std. Deviation			1.51	0.53	0.85	0.77	0.02	2.19
		Std. Error of Mean			0.42	0.15	0.24	0.21	0.01	0.61
Total	N		24	24	24	24	24	24	24	24
	Mean				51.29	18.30	-16.82	10.75	3.27	4.64
	Std. Deviation				1.52	0.56	1.01	0.73	0.02	2.93
	Std. Error of Mean				0.31	0.11	0.21	0.15	0.00	0.60

5.3 BMD Data Set Variation Between Males and Females

Within each site, I employed an independent-samples T test to investigate differences in mean BMD between the sexes. The results of the independent-samples T test showed that BMD significantly differed between males and females at the sites of S. J. de Almedina ($p = 0.002$) and Cacela ($p = 0.021$), but not at the site of the Convent of S. F. ($p = 0.643$) (Table 12).

Table 7. Values expressing collagen quality indicators, stable isotope ratios, and element sampled at the Convent de São Francisco.

Sex	Total		ID Number	Element Sampled	Conc C (%)	Conc N (%)	$\delta^{13}C‰$	$\delta^{15}N‰$	C:N Ratio	% Collagen Yield
Female			SF019	Rib	53.02	19.16	-17.27	12.69	3.23	16.40
			SF024	Rib	52.36	18.98	-16.72	14.21	3.22	16.18
			SF030	Rib	53.55	19.27	-18.43	12.49	3.24	15.05
			SF031	Rib	52.90	19.20	-18.02	11.48	3.21	14.93
			SF035	Rib	51.76	18.75	-15.73	13.13	3.22	14.52
			SF062	Rib	50.09	17.79	-17.71	13.57	3.29	1.78
			SF067	Rib	52.86	19.18	-17.87	12.06	3.22	13.15
			SF091	Rib	54.30	19.60	-17.90	13.72	3.23	18.55
			SF093	Rib	52.29	18.96	-16.91	13.59	3.22	17.57
			SF101	Rib	51.80	18.70	-18.54	12.88	3.23	13.85
			SF109	Rib	53.30	19.27	-17.30	12.32	3.23	16.81
			SF113	Rib	49.02	17.64	-17.87	11.98	3.24	4.95
			SF124	Rib	51.65	18.80	-17.80	12.76	3.21	4.16
			SF129	Rib	52.34	19.05	-18.74	11.99	3.21	12.39
			SF131	Rib	49.54	17.82	-17.92	12.35	3.24	4.39
			SF132	Rib	51.02	18.40	-18.40	11.74	3.24	5.20
			SF136	Rib	49.85	17.80	-18.39	12.41	3.27	4.50
	Total	N	17	17	17	17	17	17	17	17
		Mean			51.86	18.73	-17.74	12.67	3.23	11.43
		Std. Deviation			1.51	0.61	0.76	0.76	0.02	5.78
		Std. Error of Mean			0.37	0.15	0.19	0.18	0.01	1.40
Male			SF023	Rib	53.70	19.55	-16.41	13.94	3.21	15.07
			SF037	Rib	52.82	19.13	-17.93	13.11	3.22	16.41
			SF040	Rib	51.27	18.62	-18.04	12.68	3.21	14.51
			SF045	Rib	53.09	19.18	-17.66	13.12	3.23	12.61
			SF065	Rib	48.82	17.40	-18.28	11.98	3.27	2.03
			SF071	Rib	53.42	19.08	-17.45	14.45	3.27	12.45
			SF072	Rib	52.95	19.28	-16.98	14.12	3.20	16.18
			SF076	Rib	50.90	18.40	-17.66	12.42	3.23	7.69
			SF088	Rib	51.42	18.83	-17.74	13.12	3.19	11.74
			SF103	Rib	54.03	19.37	-17.94	13.67	3.26	16.50
			SF107	Rib	53.16	19.37	-18.02	13.89	3.20	17.06
			SF118	Prx. Phalanx	49.09	17.49	-18.08	12.22	3.28	2.65
			SF120	Rib	52.17	18.86	-17.90	12.74	3.23	11.29
	Total	N	13	13	13	13	13	13	13	13
		Mean			52.06	18.81	-17.70	13.19	3.23	12.01
		Std. Deviation			1.68	0.69	0.51	0.77	0.03	5.04
		Std. Error of Mean			0.47	0.19	0.14	0.21	0.01	1.40
Total	N		30	30	30	30	30	30	30	30
	Mean				51.95	18.76	-17.72	12.89	3.23	11.69
	Std. Deviation				1.56	0.64	0.66	0.80	0.02	5.39
	Std. Error of Mean				0.28	0.12	0.12	0.15	0.00	0.98

Table 8. Values expressing collagen quality indicators, stable isotope ratios, and element sampled at Cacela-a-Velha.

Sex	Total		ID Number	Element Sampled	Conc C (%)	Conc N (%)	$\delta^{13}C‰$	$\delta^{15}N‰$	C:N Ratio	% Collagen Yield
Female			CA001	Rib	49.88	17.90	-17.52	11.42	3.25	6.96
			CA010	Rib	53.67	18.84	-17.43	11.67	3.32	13.12
			CA015	Rib	52.63	18.42	-18.58	9.95	3.33	7.36
			CA025	Rib	51.24	18.12	-17.74	10.10	3.30	8.91
			CA028	Rib	50.29	18.05	-17.91	11.60	3.25	7.49
			CA029	Rib	49.82	17.80	-17.86	11.02	3.27	8.26
			CA034	Rib	49.73	18.09	-18.52	9.44	3.21	9.15
			CA048	Rib	51.34	17.90	-18.06	10.70	3.35	4.28
			CA050	Rib	51.05	18.19	-18.73	10.55	3.28	6.46
	Total	N	9	9	9	9	9	9	9	9
		Mean			51.07	18.14	-18.04	10.72	3.28	8.00
		Std. Deviation			1.35	0.32	0.47	0.78	0.05	2.41
		Std. Error of Mean			0.45	0.11	0.16	0.26	0.02	0.80
Male			CA003	Rib	47.11	16.86	-16.78	11.79	3.26	3.69
			CA004	Rib	53.10	18.64	-17.62	11.10	3.32	12.58
			CA005	Rib	52.42	18.77	-16.51	12.14	3.26	7.91
			CA006	Rib	50.35	18.03	-18.96	10.61	3.26	5.88
			CA009	Rib	52.54	18.88	-17.48	11.69	3.25	14.38
			CA011	Rib	52.18	18.70	-17.57	11.02	3.26	8.14
			CA013	Rib	50.34	17.76	-17.69	11.80	3.31	10.70
			CA017	Rib	50.10	17.92	-18.41	10.85	3.26	6.92
			CA018	Rib	49.95	17.94	-18.17	10.63	3.25	9.47
			CA019	Rib	51.00	18.26	-16.91	12.91	3.26	8.93
			CA021	Rib	50.26	17.97	-18.14	10.55	3.26	5.48
			CA022	Rib	51.40	18.47	-18.69	11.40	3.25	8.56
			CA023	Rib	48.08	17.12	-18.79	8.67	3.28	6.25
			CA026	Rib	52.31	18.74	-17.41	11.65	3.26	12.47
			CA027	Prx. Phalanx	51.49	18.32	-17.93	10.16	3.28	8.49
			CA031	Rib	48.75	17.28	-17.74	11.89	3.29	6.70
			CA033	Rib	50.84	18.64	-17.35	11.55	3.18	11.49
			CA036	Rib	51.31	18.42	-17.47	11.07	3.25	6.61
			CA044	Rib	48.62	17.35	-17.89	10.48	3.27	7.48
			CA045	Rib	52.92	19.17	-18.32	10.64	3.22	14.56
			CA046	Fibula	46.11	16.00	-16.93	11.47	3.36	2.62
			CA047	Prx. Phalanx	52.76	19.03	-17.34	11.66	3.24	14.22
			CA049	Rib	50.77	18.19	-15.73	13.08	3.26	5.98
			CA053	Rib	49.78	17.92	-18.45	10.04	3.24	11.33
			CA055	Rib	50.35	17.92	-17.14	13.11	3.28	5.88
	Total	N	25	25	25	25	25	25	25	25
		Mean			50.59	18.09	-17.66	11.28	3.26	8.67
		Std. Deviation			1.81	0.74	0.76	1.00	0.03	3.30
		Std. Error of Mean			0.36	0.15	0.15	0.20	0.01	0.66
Total	N		34	34	34	34	34	34	34	34
	Mean				50.72	18.11	-17.76	11.13	3.27	8.49
	Std. Deviation				1.70	0.65	0.71	0.97	0.04	3.07
	Std. Error of Mean				0.29	0.11	0.12	0.17	0.01	0.53

Table 9. Values expressing individual diagnosis for osteoporosis, BMD, and stable isotope ratio at São João de Almedina.

Sex	Total		ID Number	Diagnosis	BMD (g/cm²)	δ¹³C‰	δ¹⁵N‰
Female			AL001	Normal	.98	-16.90	10.82
			AL004	Osteoporotic	.55	-16.87	10.33
			AL005	Normal	.92	-16.19	11.20
			AL006	Osteopenic	.65	-15.15	10.64
			AL011	Osteopenic	.68	-15.84	10.33
			AL012	Normal	.99	-14.46	10.33
			AL015	Osteopenic	.72	-17.54	11.44
			AL018	Normal	.89	-17.23	10.94
			AL029	Normal	.81	-18.83	10.71
			AL031	Normal	1.01	-16.77	11.96
	Total	N	10	10	10	10	10
		Mean			.82	-16.58	10.87
		Std. Deviation			.16	1.24	.54
		Std. Error of Mean			.05	.39	.17
Male			AL007	Normal	1.25	-16.45	11.10
			AL008	Normal	.95	-17.67	9.36
			AL009	Normal	1.12	-16.42	10.99
			AL013	Normal	1.10	-15.80	10.87
			AL014	Normal	.97	-17.66	9.66
			AL017	Normal	1.04	-17.01	10.73
			AL023	Normal	1.03	-16.60	10.75
			AL024	Normal	1.02	-17.19	10.51
			AL030	Normal	1.07	-15.35	10.97
	Total	N	9	9	9	9	9
		Mean			1.06	-16.68	10.55
		Std. Deviation			.09	.79	.62
		Std. Error of Mean			.03	.26	.21
Total	N		19	19	19	19	19
	Mean				.93	-16.63	10.72
	Std. Deviation				.18	1.02	.58
	Std. Error of Mean				.04	.24	.13

At all sites, mean $\delta^{13}C$ is similar between males and females (within 0.1‰) and any differences were not statistically significant. For $\delta^{15}N$, at each site, males have a higher mean (approximately 0.4‰) but these differences are not statistically significant (Table 9, Table 10, Table 11).

5.4 BMD Data Set Variation by Site

One-way ANOVA analysis was employed to investigate whether variable means differed by site. Means for $\delta^{15}N$, $\delta^{13}C$, and BMD were compared across the three sites separately by sex. Mean BMD varies significantly for males ($F(2,31) = 10.455$, $p = 0.000$) but not for females ($F(2, 28) =$

Table 10. Values expressing individual diagnosis for osteoporosis, BMD, and stable isotope ratio at the Convento de São Francisco.

Sex	Total		ID Number	Diagnosis	BMD (g/cm²)	δ¹³C‰	δ¹⁵N‰
Male			SF023	Normal	.92	-16.41	13.94
			SF037	Normal	.95	-17.93	13.11
			SF045	Normal	.90	-17.66	13.12
			SF065	Osteopenic	.67	-18.28	11.98
			SF072	Osteoporotic	.57	-16.98	14.12
			SF088	Osteopenic	.66	-17.74	13.12
			SF103	Normal	.81	-17.94	13.67
			SF118	Normal	.99	-18.08	12.22
	Total	N	8	8	8	8	8
		Mean			.81	-17.63	13.16
		Std. Deviation			.16	.63	.76
		Std. Error of Mean			.06	.22	.27
Female			SF024	Normal	.74	-16.72	14.21
			SF031	Normal	.99	-18.02	11.48
			SF035	Osteoporotic	.53	-15.73	13.13
			SF067	Normal	.96	-17.87	12.06
			SF091	Normal	.84	-17.90	13.72
			SF093	Normal	.90	-16.91	13.59
			SF109	Osteopenic	.62	-17.30	12.32
			SF124	Normal	.96	-17.80	12.76
			SF131	Osteopenic	.68	-17.92	12.35
			SF132	Osteopenic	.69	-18.40	11.74
			SF136	Osteopenic	.65	-18.39	12.41
	Total	N	11	11	11	11	11
		Mean			.78	-17.54	12.70
		Std. Deviation			.16	.81	.86
		Std. Error of Mean			.05	.24	.26
Total	N		19	19	19	19	19
	Mean				.79	-17.58	12.90
	Std. Deviation				.16	.72	.83
	Std. Error of Mean				.04	.17	.19

2.078, p = 0.144). Mean $\delta^{13}C$ also varies significantly between sites for males (F(2,31) = 4.968, p = 0.013), but not for females (F(2, 28) = 2.436, p = 0.106). Lastly, the variable $\delta^{15}N$ varies significantly among sites for both males (F(2, 31) = 18.468, p = 0.000) and females (F(2, 28) = 23.71, p = 0.000) (Table 13).

Post hoc comparisons using the Tukey multiple comparison test indicates that, for all pairs of sites, the mean scores are significantly different for BMD, $\delta^{13}C‰$, and $\delta^{15}N‰$. At the site of the Convent of SF males differ significantly in BMD (M = 0.81, SD = 0.16), from those at the other two sites, exhibiting the lowest values for males across all three sites. The Convent of SF also illustrates a significant difference from the other two sites in both males (M = 13.16, SD = 0.76) and

Table 11. Values expressing individual diagnosis for osteoporosis, BMD, and stable isotope ratio at Cacela-a-Velha.

Sex	Total		ID Number	Diagnosis	BMD (g/cm²)	δ¹³C‰	δ¹⁵N‰
Female			CA001	Normal	.87	-17.52	11.42
			CA010	Normal	.73	-17.43	11.67
			CA025	Normal	.96	-17.74	10.10
			CA028	Normal	.83	-17.91	11.60
			CA029	Normal	.78	-17.86	11.02
			CA034	Normal	1.07	-18.52	9.44
			CA048	Normal	.93	-18.06	10.70
			CA050	Normal	1.12	-18.73	10.55
	Total	N	8	8	8	8	8
		Mean			.91	-17.97	10.81
		Std. Deviation			.14	.45	.78
		Std. Error of Mean			.05	.16	.27
Male			CA003	Normal	.91	-16.78	11.79
			CA005	Normal	1.25	-16.51	12.14
			CA006	Normal	1.08	-18.96	10.61
			CA009	Normal	.91	-17.48	11.69
			CA017	Normal	1.00	-18.41	10.85
			CA018	Normal	1.26	-18.17	10.63
			CA021	Normal	1.13	-18.14	10.55
			CA023	Normal	1.02	-18.79	8.67
			CA026	Normal	1.07	-17.41	11.65
			CA031	Normal	.88	-17.74	11.89
			CA033	Normal	1.06	-17.35	11.55
			CA036	Normal	.91	-17.47	11.07
			CA045	Normal	1.02	-18.32	10.64
			CA047	Normal	1.02	-17.34	11.66
			CA049	Normal	1.24	-15.73	13.08
			CA053	Normal	.90	-18.45	10.04
			CA055	Normal	.91	-17.14	13.11
	Total	N	17	17	17	17	17
		Mean			1.03	-17.66	11.27
		Std. Deviation			.13	.85	1.09
		Std. Error of Mean			.03	.21	.26
Total	N		25	25	25	25	25
	Mean				.99	-17.76	11.12
	Std. Deviation				.14	.75	1.01
	Std. Error of Mean				.03	.15	.20

females (M = 12.70, SD = 0.86) for δ^{15}N‰ means, whereas its males and females that exhibit the highest δ^{15}N‰ values across all three sites. In males (M = -16.68, SD = 0.79), δ^{13}C‰ means differ significantly for the site of SJ de Almedina, indicating that they exhibit the lowest values compared to the other sites.

Table 12. Independent-samples T test between males and females for the BMD data set.

	BMD			$\delta^{13}C$			$\delta^{15}N$		
Site	t value	df	*p*	t value	df	*p*	t value	df	*p*
SJ de Almedina	-4.065	14.379	0.001	0.22	17	0.828	1.206	17	0.244
Convent of SF	-0.423	17	0.677	0.248	17	0.807	-1.182	17	0.253
Cacela	-2.163	23	0.041	-0.971	23	0.342	-1.068	23	0.297

Table 13. One-way ANOVA test results investigating difference in variables between all sites and by sex for the BMD data set.

	Males				Females			
Variable	df between	df within	F	*p*	df between	df within	F	*p*
BMD	2	31	10.455	0.000	2	28	2.078	0.144
δ13C	2	31	4.968	0.013	2	28	2.436	0.106
δ15N	2	31	18.468	0.000	2	28	23.71	0.000

5.5 Variation by Disease Status

Normal and diseased (osteopenic and osteoporotic) individuals were compared by age and sex groups (Table 14, Table 15, and Table 16). However, sample sizes were too small for statistical analysis of mean difference in isotope values among the various groups; a sample size of at least 9 for each subgroup is required to detect a difference of one per mil, assuming population standard deviation is around 0.7 per mil and the common acceptable power of 80% (Pechenkina, Ambrose, Xiaolin, & Benfer, 2005). The Cacela-a-Velha samples yielded no diseased individuals; however, information comparing its subjects' $\delta^{15}N$ and $\delta^{13}C$ values by age and sex are still presented (Table 16).

Table 14. Comparison of normal and diseased individuals by age (years) and sex: São João de Almedina.

São João de Almedina

	Female					Male				
		d13C (0/00)		d15N (0/00)			d13C (0/00)		d15N (0/00)	
YRS	N	Mean	SD	Mean	SD	N	Mean	SD	Mean	SD
Normal										
20-29	-	-	-	-	-	1	-17.01	-	10.73	-
30-49	4	-17.26	1.13	11.2	0.54	3	-16.48	1.16	10.44	0.94
50+	2	-15.68	1.72	10.58	0.35	5	-16.74	0.71	10.58	0.56
Osteopenic/Osteoporotic										
20-29	-	-	-	-	-	-	-	-	-	-
30-49	1	-17.54	-	11.44	-	-	-	-	-	-
50+	3	-15.95	0.87	10.43	0.18	-	-	-	-	-

Table 15. Comparison of normal and diseased individuals by age (years) and sex: Convento de São Francisco.

Convento de São Francisco

	Female					Male				
		d13C (0/00)		d15N (0/00)			d13C (0/00)		d15N (0/00)	
YRS	N	Mean	SD	Mean	SD	N	Mean	SD	Mean	SD
Normal										
20-29	2	-17.89	0.02	12.89	1.17	-	-	-	-	-
30-49	-	-	-	-	-	2	-18.01	0.11	12.67	0.63
50+	4	-17.36	0.64	13.01	1.18	3	-17.34	0.81	13.58	0.42
Osteopenic/Osteoporotic										
20-29	1	-18.4	-	11.74	-	1	-18.28	-	11.98	-
30-49	4	-17.34	1.16	12.55	0.39	1	-17.74	-	13.12	-
50+	-	-	-	-	-	1	-16.98	-	14.12	-

Table 16. Comparison of individuals by age (years) and sex: Cacela-a-Velha.

Cacela-a-Velha

	Female					Male				
		d13C (0/00)		d15N (0/00)			d13C (0/00)		d15N (0/00)	
YRS	N	Mean	SD	Mean	SD	N	Mean	SD	Mean	SD
Normal										
20-29	3	-17.71	0.17	10.85	0.68	6	-17.56	0.9	11.32	0.64
30-49	3	-18.02	0.66	11.27	0.63	6	-17.48	1.08	11.37	1.74
50+	2	-18.29	0.33	10.07	0.89	5	-17.99	0.44	11.1	0.59
Osteopenic/Osteoporotic										
20-29	-	-	-	-	-	-	-	-	-	-
30-49	-	-	-	-	-	-	-	-	-	-
50+	-	-	-	-	-	-	-	-	-	-

5.6 Bone Mineral Density vs. $\delta^{13}C$ and $\delta^{15}N$

The primary goal of this study is to investigate the relationship between dietary protein source and BMD. As discussed, simple linear regression analysis was used to investigate the form, strength, and significance of the relationship between BMD and the two independent diet variables ($\delta^{13}C$ and $\delta^{15}N$). Regression analysis was conducted by sex for each site. Scatterplots of variable pairs are shown in Figure 11, Figure 12, Figure 13, Figure 14, Figure 15, Figure 16, Figure 17, Figure 18, Figure 19, Figure 20, Figure 21, Figure 22, and regression equations are shown in Table 17.

Most of the regression analyses indicate no linear relationship between BMD and either $\delta^{13}C$ or $\delta^{15}N$. However, two of the subsamples – the SJ de Almedina males and Cacela females – exhibit statistically significant relationships between $\delta^{15}N$ and BMD values, and the Cacela females also exhibit a significant relationship between $\delta^{13}C$ and BMD. For the SJ de Almedina males, the coefficient of determination (R^2) indicates that 60% of the variation in BMD is explained by variation in $\delta^{15}N$ (Figure 12, Table 13). This is a positive correlation, indicating that as $\delta^{15}N$ increases so does BMD. Interestingly, females at Cacela exhibit a negative correlation between BMD and $\delta^{15}N$, indicating the opposite relationship that as $\delta^{15}N$ increases, the density of bone in turn decreases (Figure 22, Table 17). The coefficient of determination (R^2) indicates that 62% of the

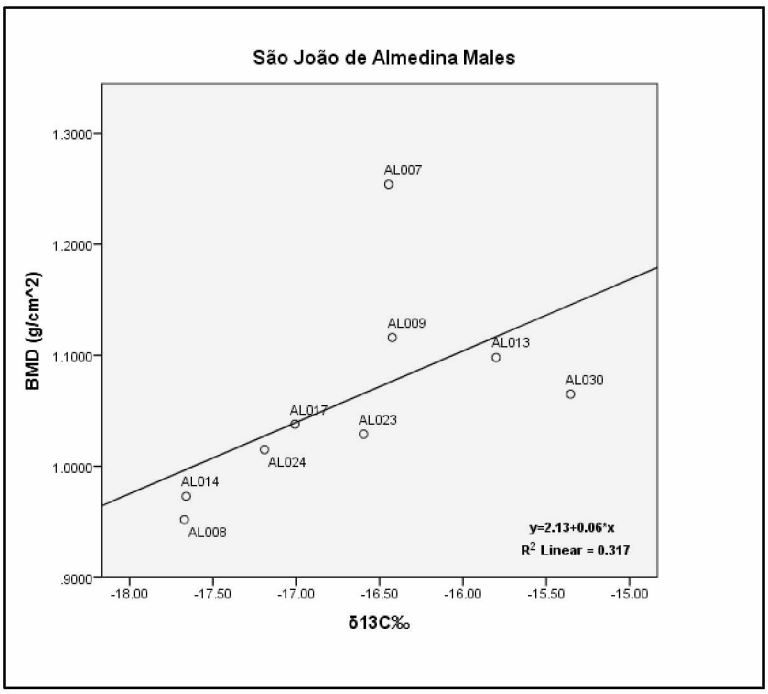

Figure 11. Scatter plot with line of best fit between BMD and δ^{13}C‰ in SJ de Almedina males.

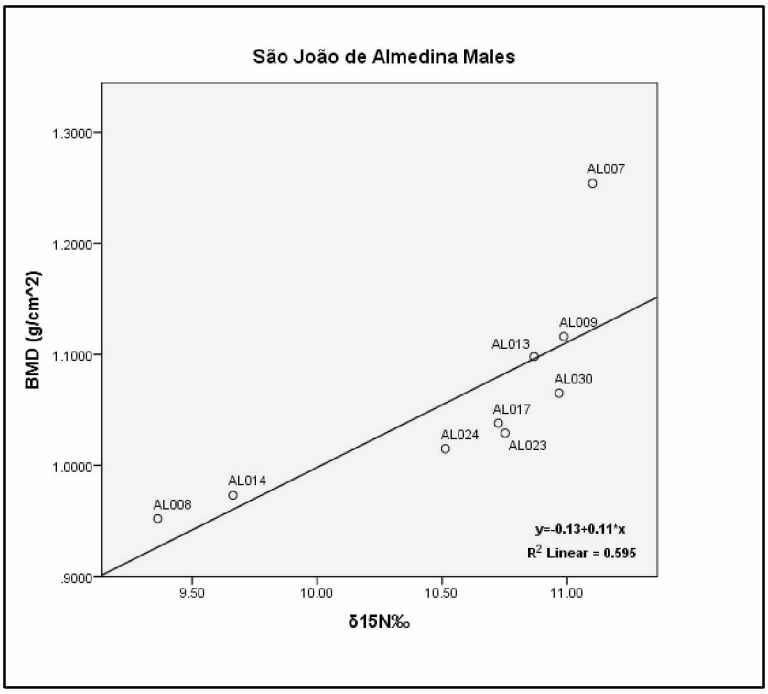

Figure 12. Scatter plot with line of best fit between BMD and δ^{15}N‰ in SJ de Almedina males.

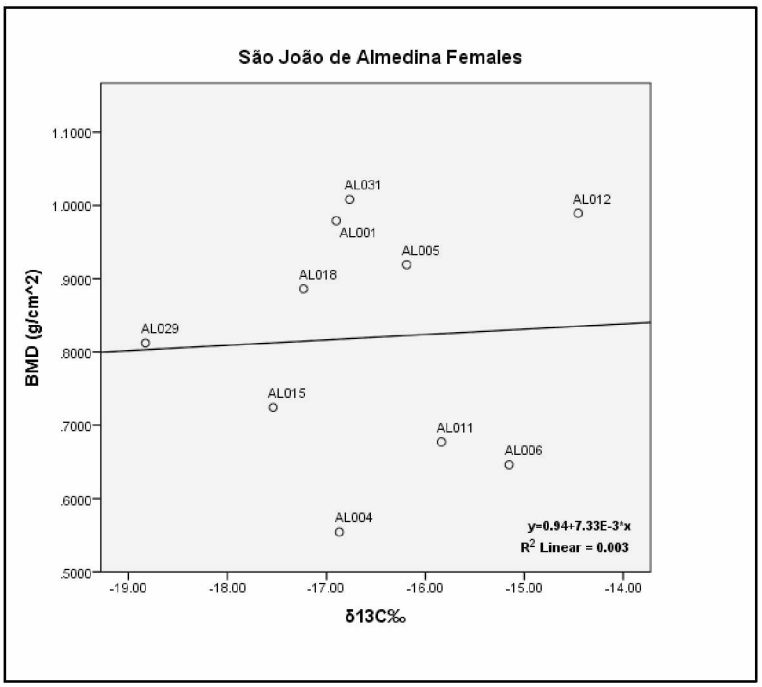

Figure 13. Scatter plot with line of best fit between BMD and δ^{13}C‰ in SJ de Almedina females.

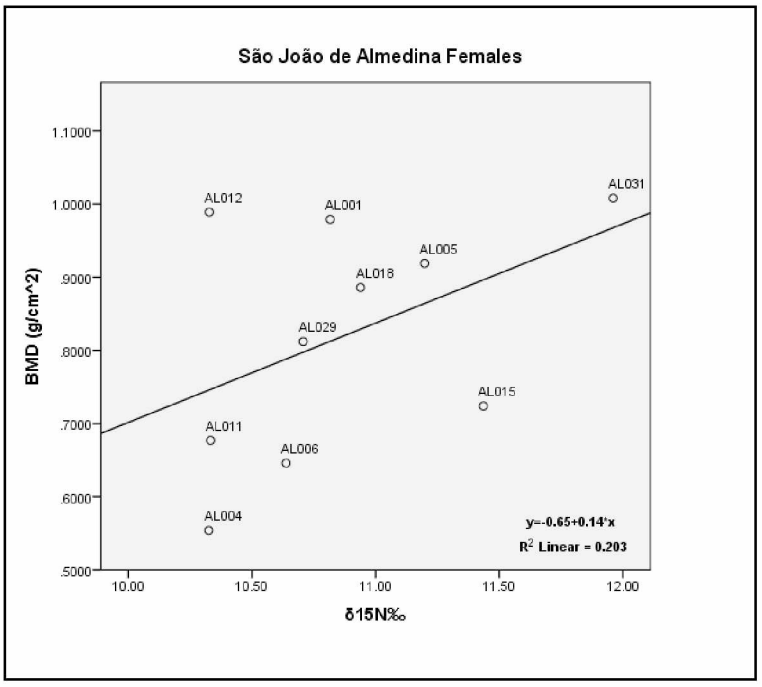

Figure 14. Scatter plot with line of best fit between BMD and δ^{15}N‰ in SJ de Almedina females.

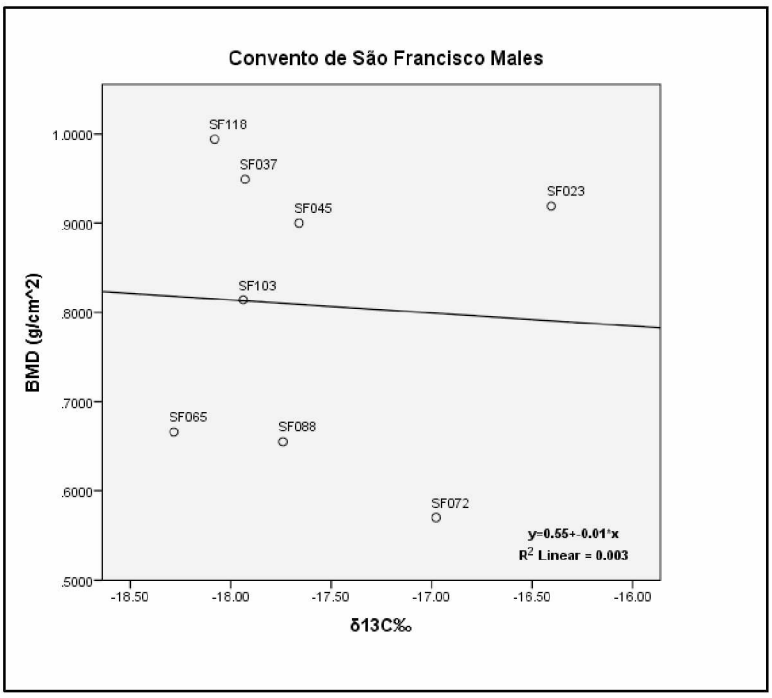

Figure 15. Scatter plot with line of best fit between BMD and δ^{13}C‰ in the convent of SF males.

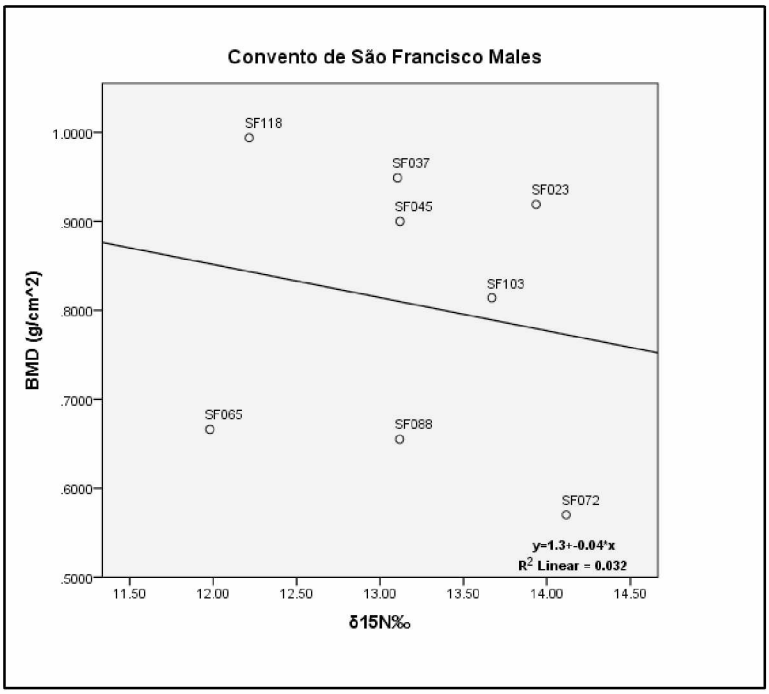

Figure 16. Scatter plot with line of best fit between BMD and δ^{15}N‰ in the convent of SF males.

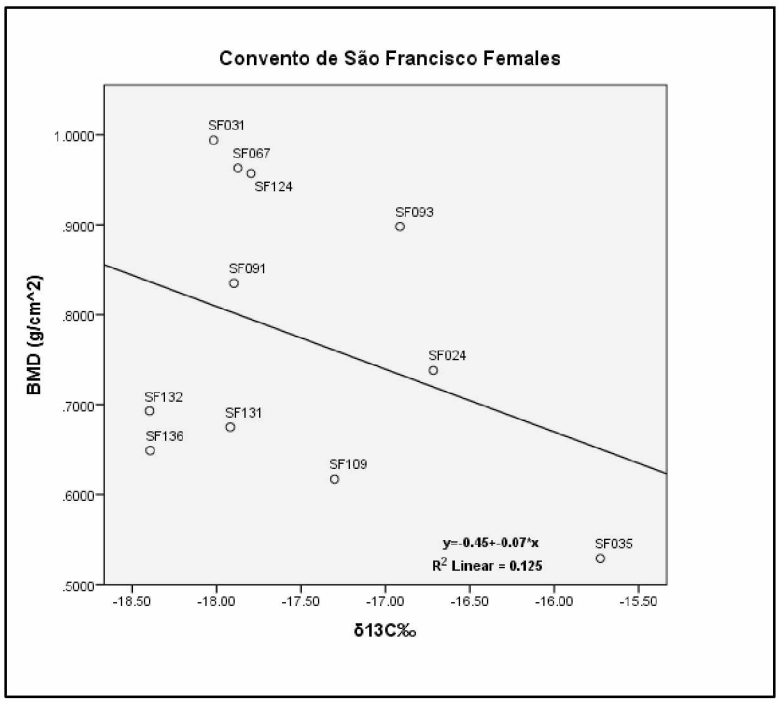

Figure 17. Scatter plot with line of best fit between BMD and $\delta^{13}C‰$ in the convent of SF females.

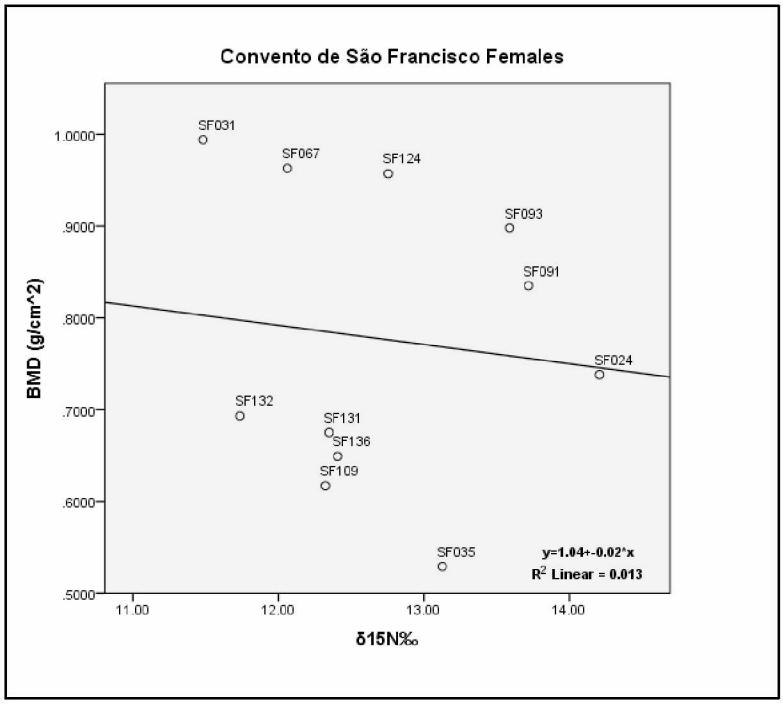

Figure 18. Scatter plot with line of best fit between BMD and $\delta^{15}N‰$ in the convent of SF females.

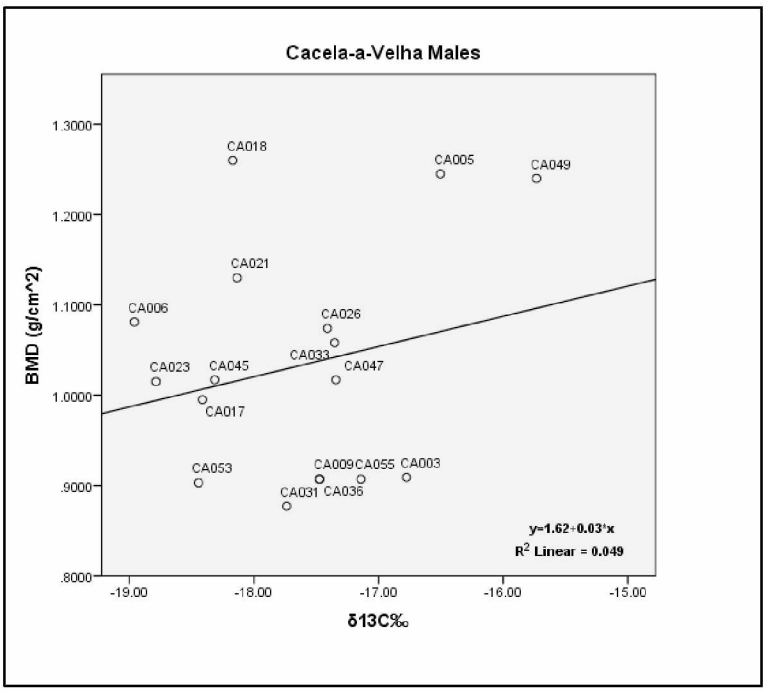

Figure 19. Scatter plot with line of best fit between BMD and δ^{13}C‰ in Cacela males.

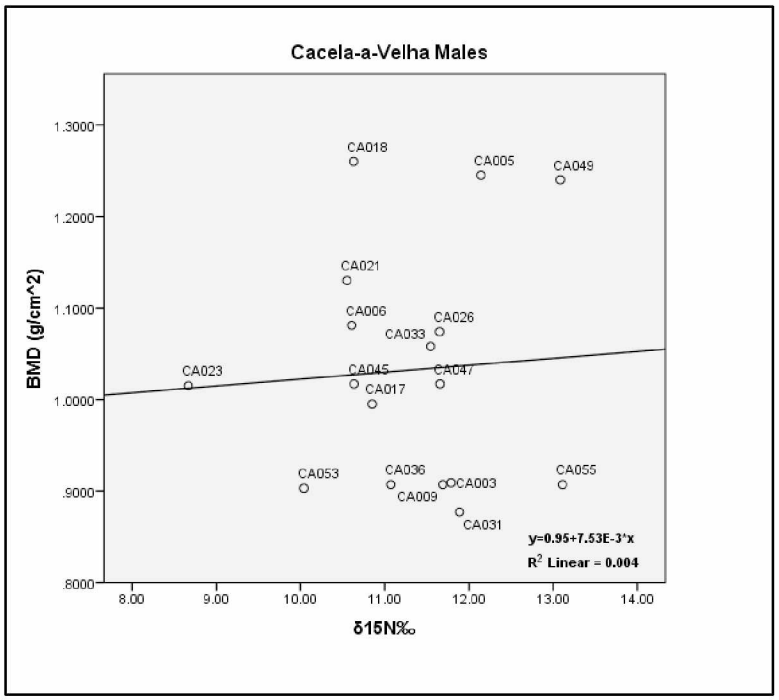

Figure 20. Scatter plot with line of best fit between BMD and δ^{15}N‰ in Cacela males.

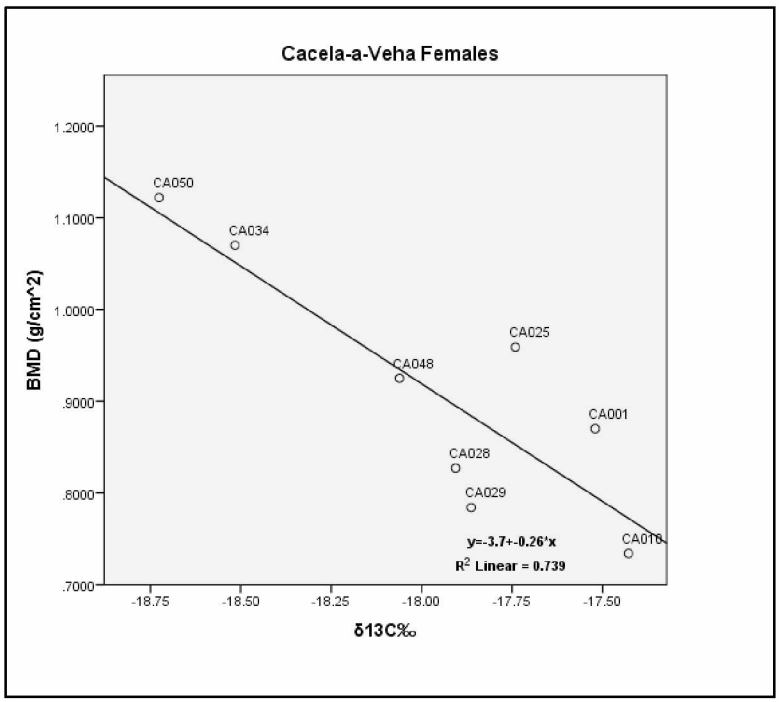

Figure 21. Scatter plot with line of best fit between BMD and $\delta^{13}C‰$ in Cacela females.

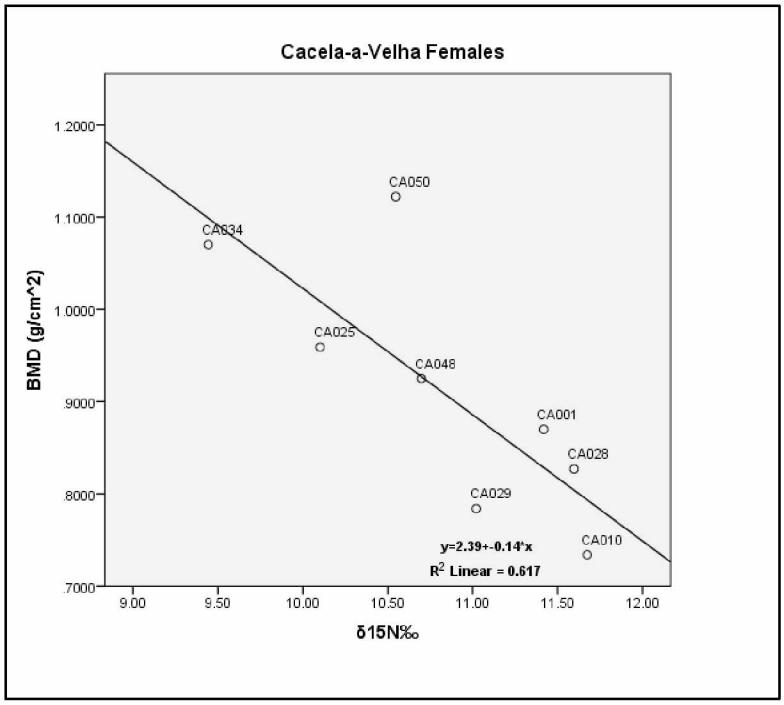

Figure 22. Scatter plot with line of best fit between BMD and $\delta^{15}N‰$ in Cacela females.

Table 17. Simple linear regression results investigating relationship between the predictor stable isotope values and response BMD values.

	Predictor variable (x) δ13C‰; Response variable (y) BMD							
	Males				Females			
Site	R^2	Std. error of estimate	Regression equation	*p*	R^2	Std. error of estimate	Regression equation	*p*
SJ de Almedina	0.317	0.079	y=2.13+0.06*x	0.115	0.003	0.171	y=0.94+7.33E-3*x	0.877
Convent of SF	0.003	0.171	y=0.55+-0.01*x	0.892	0.125	0.157	y=-0.45+-0.07*x	0.286
Cacela	0.049	0.128	y=1.62+0.03*x	0.391	0.739	0.075	y=-3.7+-0.26*x	0.006
	Predictor variable (x) δ15N‰; Response variable (y) BMD							
	Males				Females			
Site	R^2	Std. error of estimate	Regression equation	*p*	R^2	Std. error of estimate	Regression equation	*p*
SJ de Almedina	0.595	0.061	y=-0.13+0.11*x	0.015	0.203	0.153	y=-0.65+0.14*x	0.191
Convent of SF	0.032	0.168	y=1.3+-0.04*x	0.671	0.013	0.167	y=1.04+-0.02*x	0.74
Cacela	0.004	0.131	y=0.95+7.53E-3*x	0.807	0.617	0.091	y=2.39+-0.14*x	0.021

variation in bone mineral density is explained by variation in $\delta^{15}N$. Also exhibited in Cacela females is a negative correlation between $\delta^{13}C$ and BMD, a statistically significant relationship ($p = 0.006$) for which the coefficient of determination (R^2) indicates that 74% of the variation in BMD is explained by variation in $\delta^{13}C$ (Figure 21, Table 17).

These results suggest a complicated relationship between protein source and OP risk; this will be discussed in more detail in the following chapter.

5.7 Dietary Modeling Results

Dietary modeling was based on stable isotope ratios for each site and by sex, in addition to stable isotope ratio means from outside studies. At SJ de Almedina mean $\delta^{13}C$ values equal -16.82‰ and range from -18.83 to -14.4‰, while mean $\delta^{15}N$ values equal 10.75‰ and range from 8.92 to 12.12‰. At the Convent SF, mean $\delta^{13}C$ values equal -17.72‰ and range from -18.74 to -15.73‰, while mean $\delta^{15}N$ values equal 12.89‰ and range from 11.98 to 14.45 ‰. At Cacela, mean $\delta^{13}C$ values equal -17.76‰ and range from -18.74 to -15.73‰, while mean $\delta^{15}N$ values equal 11.13‰ and range from 8.67 to 13.11 ‰. The means for each study site and sex are presented in Table 6, Table 7, and Table 8 and illustrated graphically in Figure 23.

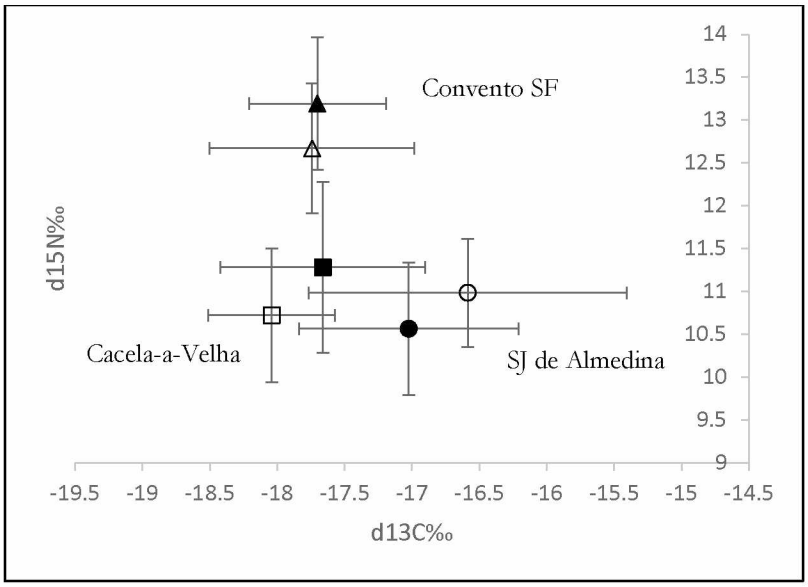

Figure 23. Comparison of δ^{15}N‰ and δ^{13}C‰ values (means and standard deviations) for males (filled) and females (unfilled) by site.

5.8 Diet Modeling Data Set Variation Between Males and Females

Within each site, I employed the independent-samples T test to investigate differences in mean dietary variables (δ^{13}C‰ and δ^{15}N‰) between the sexes. The results showed that mean δ^{13}C is similar between males and females at all three sites. For δ^{15}N, there are no statistically significant differences between males and females at any of the sites (Table 18). However, there is a 0.4‰ difference in both δ^{13}C‰ and δ^{15}N‰ between males and females at each site that is not statistically significant (Figure 23).

Table 18. Independent-samples T test for all dietary values between males and females for the diet modeling data set.

	δ^{13}C			**δ^{15}N**		
Site	t value	df	*p*	t value	df	*p*
SJ de Almedina	0.22	17	0.828	1.206	17	0.244
Convent of SF	-0.153	28	0.879	-1.838	28	0.077
Cacela	-1.409	32	0.169	-1.524	32	0.137

5.9 Diet Modeling Data Set Variation by Site

One-way ANOVA analysis was employed to investigate whether variable means within the diet modeling data set differed by site. Mean $\delta^{15}N$ and $\delta^{13}C$ were compared across the three sites separately by sex. Mean $\delta^{13}C$ varies significantly among sites for both males ($p = 0.029$) and females ($p = 0.022$) (Table 19). Post hoc comparisons using the Tukey multiple comparison test indicated that the mean scores for each pair of sites are significantly different for $\delta^{13}C‰$. Males differ the greatest in mean $\delta^{13}C‰$ value between SJ de Almedina (M = -17.02, SD = 0.24) and Cacela (M = -17.66, SD = 0.76), with SJ de Almedina exhibiting the lowest mean value and Cacela the highest. Female mean $\delta^{13}C‰$ values (M = -16.58, SD = 1.18) indicate SJ de Almedina as the outlier, exhibiting the lowest values when compared to the other two sites.

Table 19. One-way ANOVA test results investigating difference in dietary variables between all sites and by sex for the diet modeling data set.

	Males				Females			
Variable	df between	df within	F	*p*	df between	df within	F	*p*
δ13C	2	48	3.827	0.029	2	36	4.235	0.022
δ15N	2	48	30.949	0.000	2	36	30.988	0.000

Mean $\delta^{15}N$ also varies significantly among sites, for both males ($p < 0.001$) and females ($p = 0.000$) (Table 19). Post hoc comparisons using the Tukey multiple comparison test indicated that the mean scores for each of the three sites are significantly different for $\delta^{15}N‰$. The Convent of SF illustrates a significant difference in both males (M = 13.19, SD = 0.77) and females (M = 12.67, SD, = 0.76) among the three sites; both males and females exhibit the highest mean values in $\delta^{15}N‰$ when compared to the other two sites. Figure 23 illustrates the mean values of $\delta^{13}C$ and $\delta^{15}N$ for males and females at each site.

5.10 Diet Modeling

To determine the general dietary pattern at each site by sex, simple linear regression was performed as a simple correlation analysis between carbon and nitrogen isotope ratios to investigate

significant associations between the two variables for both males and females. Correlation between $\delta^{13}C$ and $\delta^{15}N$ indicates that marine foods contributed a substantial proportion of dietary protein (see Chapter 2.9 for discussion).

Scatterplots are shown for each site in Figure 24, Figure 25, Figure 26, Figure 27, Figure 28, and Figure 29, while regression equations are shown in Table 20. These tests and scatterplots present isotopic evidence for multiple food sources and can assist discerning between marine and terrestrial protein being consumed. Because $\delta^{13}C$ and $\delta^{15}N$ values from marine protein sources tend to be higher than those of terrestrial animals, populations consuming such fish should exhibit a correlation between the two isotopes (e.g., Ambrose et al., 1997, Lubell & Jackes, 1994). Comparison with human and faunal stable isotope ratio data from other studies performed within the Iberian Peninsula is also presented later in this chapter (Table 21, Table 22, and Figure 31).

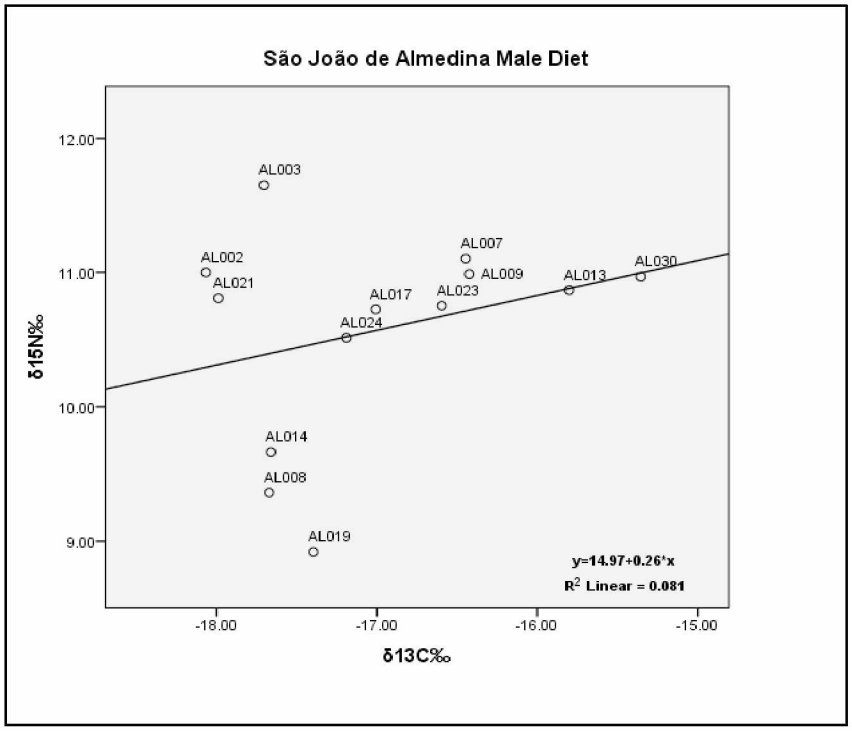

Figure 24. Scatter plot of $\delta^{13}C$ and $\delta^{15}N$ for males at SJ de Almedina.

In general, mean carbon and nitrogen stable isotope ratios from the three sites in this study are similar to values from other medieval sites on the Iberian Peninsula (Figure 30 and Table 21). Human nitrogen isotope values are elevated over those of livestock from the region, suggesting that

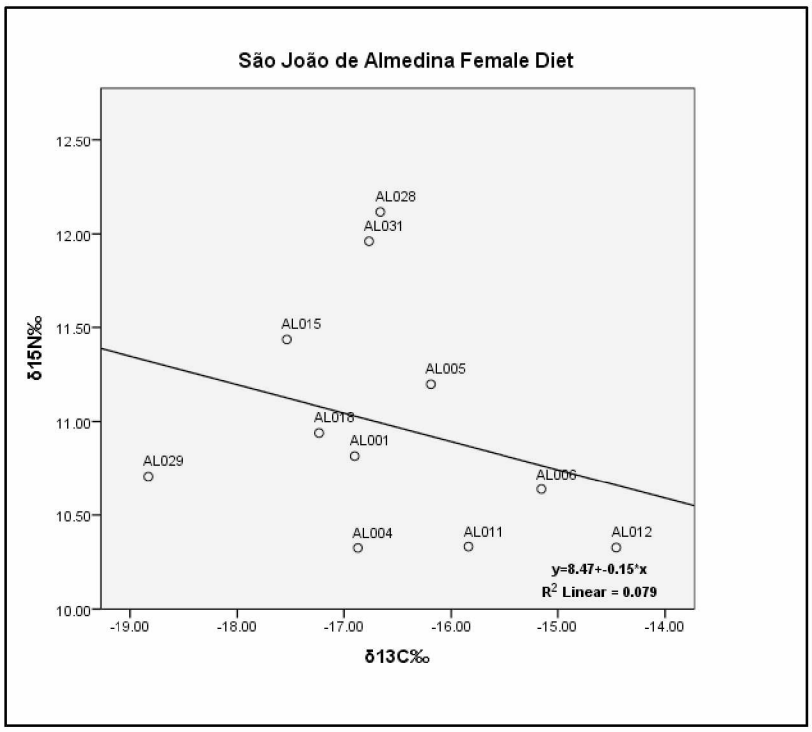

Figure 25. Scatter plot of $\delta^{13}C$ and $\delta^{15}N$ for females at SJ de Almedina.

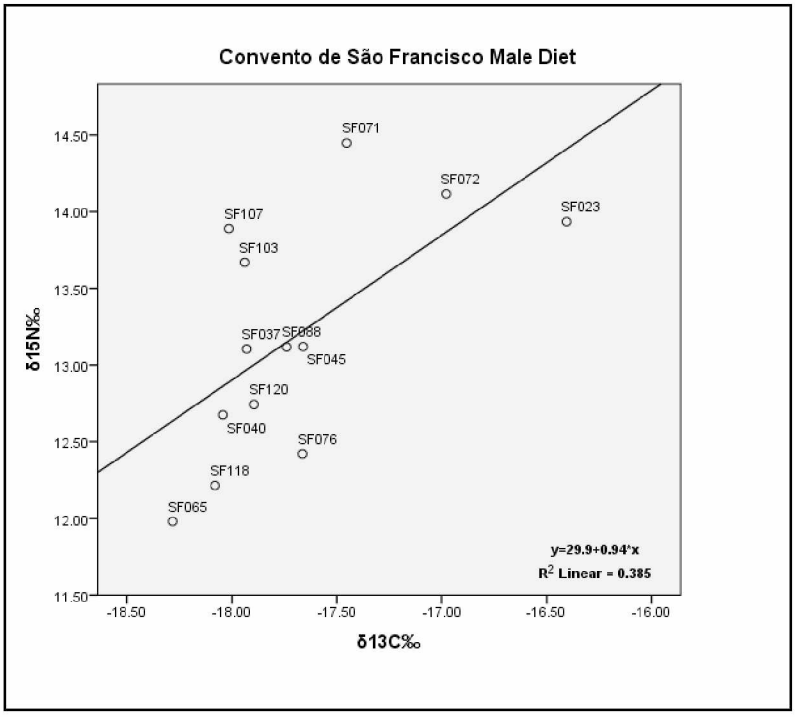

Figure 26. Scatter plot of $\delta^{13}C$ and $\delta^{15}N$ for males at the convent of SF.

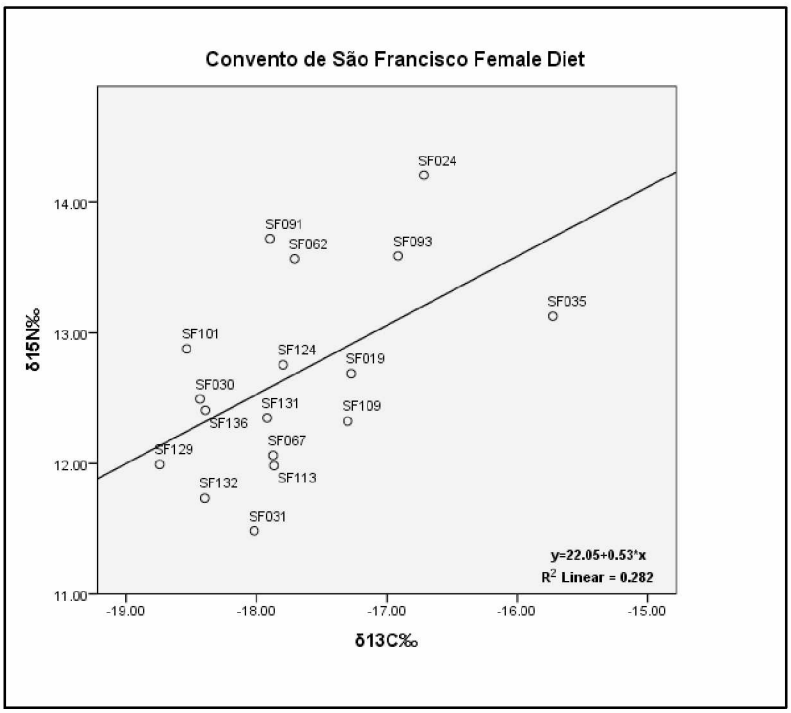

Figure 27. Scatter plot of $\delta^{13}C$ and $\delta^{15}N$ for females at the convent of SF.

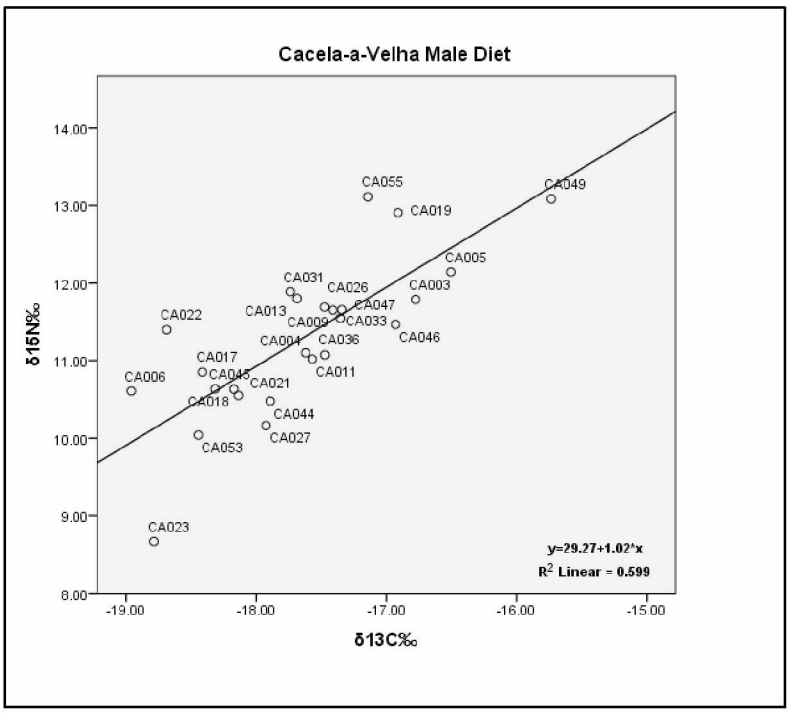

Figure 28. Scatter plot of $\delta^{13}C$ and $\delta^{15}N$ of males at Cacela-a-Velha.

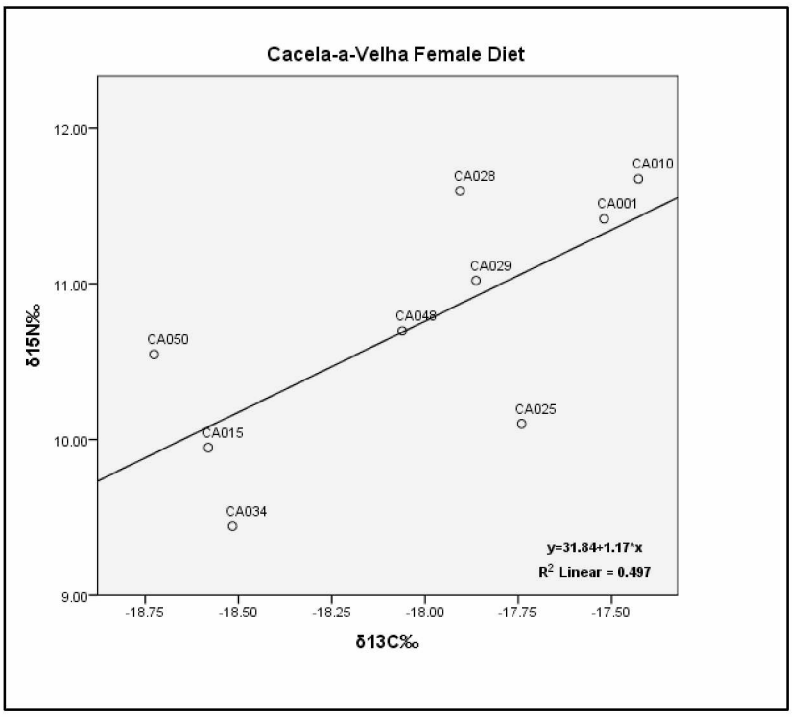

Figure 29. Scatter plot of $\delta^{13}C$ and $\delta^{15}N$ of females at Cacela-a-Velha.

Table 20. Correlation results investigating relationship between $\delta^{13}C‰$ and $\delta^{15}N‰$ values.

Site	Sex	R	Std. error of estimate	*p*
SJ de Almedina	Male	0.284	0.775	0.347
	Female	0.281	0.640	0.402
Convent of SF	Male	0.621	0.634	0.024
	Female	0.531	0.667	0.028
Cacela-a-Velha	Male	0.774	0.645	0.000
	Female	0.705	0.593	0.034

a substantial proportion of dietary protein came from non-plant sources (e.g. terrestrial animal products or fish) (Alexander et al., 2015) (Figure 31 and Table 22). Carbon isotope values are also elevated over those expected for a pure terrestrial C3 diet, suggesting that some dietary protein came

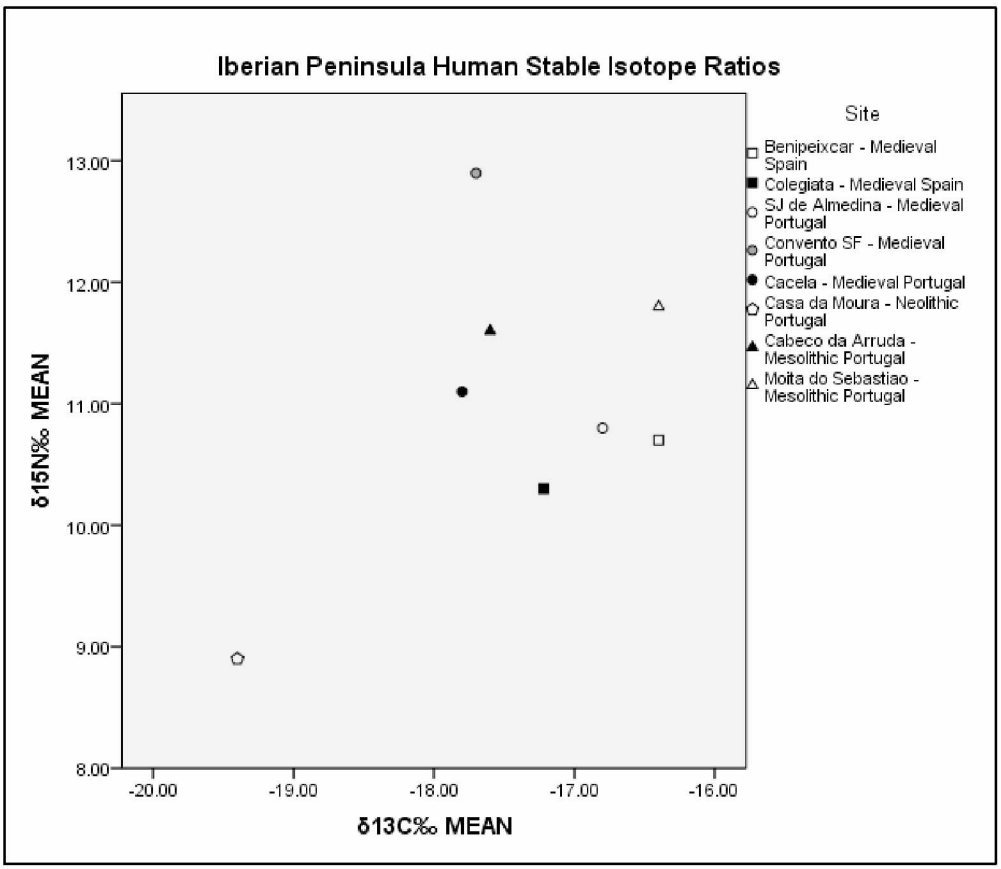

Figure 30. Mean human stable isotope values from reference sites in Spain and Portugal, including the mean human values from this study. See Table 21 for exact values.

from C4 and/or marine sources (Richards & Hedges, 1999). However, variations do exist among the three study sites.

At SJ de Almedina, means for carbon and nitrogen isotope ratios closely resemble those from the Spanish medieval site of Benipeixcar (Alexander et al., 2015). At SJ de Almedina, isotope data suggests a diet of mainly terrestrial protein. Males and females from the site of SJ de Almedina do not exhibit a correlation between $\delta^{13}C$ and $\delta^{15}N$ values (Table 20), suggesting that marine protein input to diet was minimal. Because C4 plants exhibit stable isotope ratios between the range of -9 to -16‰ and C3 plants a range of -20 to -35‰, this in turn suggests that the elevated $\delta^{13}C$ values at this site may be explained by protein derived from C4 plants (Katzenberg, 2008). Likely millet, this C4 plant could have been consumed either as a primary food source or secondarily by consuming the livestock that may have been foddered on it.

Table 21. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ (mean ± 1 standard deviation[a]) for human remains from sites on the Iberian Peninsula.

Region	Site	Time Period	n	δ13C	δ15N	Reference
Spain	Colegiata	13th - 16th C. AD	24	-17.2 ± 1.0	10.3 ± 0.8	Alexander et al. (2015)
Spain	Benipeixcar	15th - 16th C. AD	20	-16.4 ± 1.0	10.7 ± 0.6	Alexander et al. (2015)
Portugal	São João de Almedina	12th - 15th C. AD	20	-16.8 ± 1.0	10.8 ± 0.7	This study
Portugal	Convent of San Francisco	13th - 14th C. AD	30	-17.7 ± 0.7	12.9 ± 0.8	This study
Portugal	Cacela-a-Velha	13th - 16th C. AD	26	-17.8 ± 0.7	11.1 ± 1	This study
Portugal	Cabeço da Arruda	Mesolithic	5	-17.6 ± 1.5	11.6 ± 0.9	Lubell & Jackes (1994)
Portugal	Moita do Sebastião	Mesolithic	5	-16.4 ± 0.7	11.8 ± 1.1	Lubell & Jackes (1994)
Portugal	Casa da Moura	Neolithic	4	-19.4 ± 0.2	8.9 ± 0.5	Lubell & Jackes (1994)

[a] Means and standard deviations calculated from raw data presented in references, unless summary statistics were presented in the original.

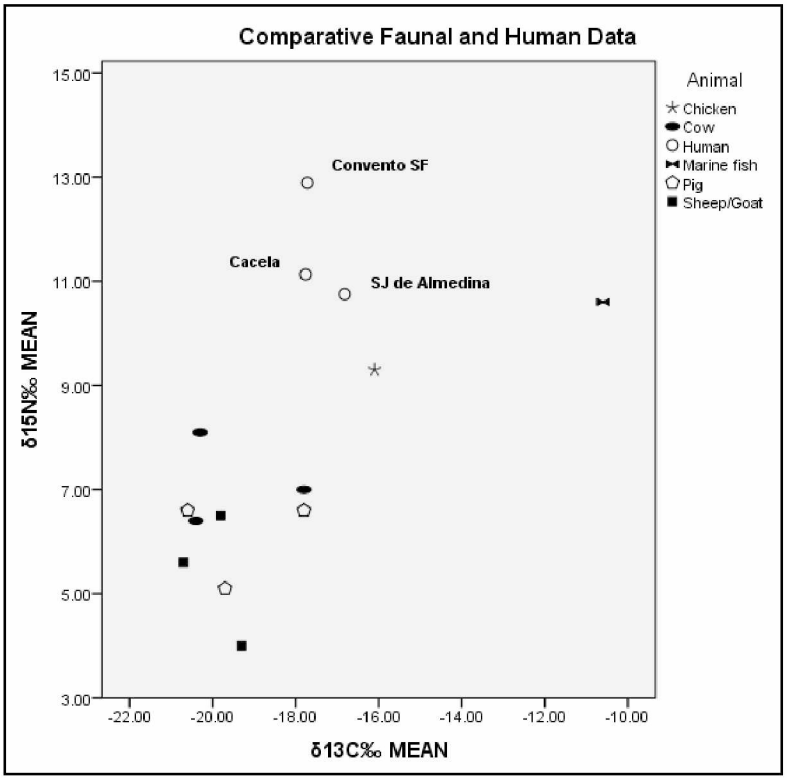

Figure 31. Mean faunal stable isotope values from reference sites in Spain and Portugal, including the mean human values from this study. See Table 2 and Table 23 for exact values.

Table 22. Bone collagen $\delta^{13}C$ and $\delta^{15}N$ (mean ± 1 standard deviation[a]) for archaeological livestock and fauna from sites on the Iberian Peninsula.

Region	Site	Time Period	Cow			Sheep/goat			Pig			Chicken			Marine Fish			Reference
			n	δ13C	δ13C	n	δ13C	δ15N	n	δ13C	δ15N	n	δ13C	δ15N	n	δ13C	δ15N	
Portugal	Zambujal	2800-1800 BC	3	-20.4 ± 0.1	6.4 ± 1.6	4	-20.7 ± 0.3	5.6 ± 0.4	7	-20.6 ± 0.6	6.6 ± 2.0	-	-	-	-	-	-	Waterman et al. (2011)
Spain	Ibiza	10th-13th C. AD	Ukn	-20.3 ± 0.1	8.1 ± 0.3	Ukn	-19.8 ± 0.7	6.5 ± 2.3	1	-19.7	5.1	-	-	-	-	-	-	Fuller et al. (2010)
Spain	Gandía (Valencia)	13th-16th C. AD	5	-17.8 ± 2.9	7.0 ± 1.2	9	-19.3 ± 0.2	4.0 ± 0.8	1	-17.8	6.6	4	-16.1 ± 2.4	9.3 ± 0.8	-	-	-	Alexander et al. (2015)
Spain	Albarracin (Teruel)	11th-12th C. AD	-	-	-	-	-	-	-	-	-	-	-	-	5	10.6 ± 0.8	10.6 ± 1.9	Alexander et al. (2015)

[a] Means and standard deviations calculated from raw data presented in references, unless summary statistics were presented in the original

At the Convent of SF, means for carbon and nitrogen isotope ratios deviate significantly from other medieval sites on the Iberian Peninsula. Specifically, nitrogen isotope ratios exceed values reported from Mesolithic Iberian Peninsula sites that are known to have consumed extensive amounts of marine protein (Lubell & Jackes, 1991). At this site, isotope data suggested a diet of both terrestrial and marine protein input. Males and females from the Convent of SF exhibit a significant correlation between $\delta^{13}C$ and $\delta^{15}N$ values (Table 20), suggesting that marine protein input to diet was significant. This in turn suggests that the somewhat elevated $\delta^{13}C$ values at this site may be explained either by the consumption of C4 plants (e.g., millet), either directly or indirectly, or by marine protein.

At the site of Cacela, means for carbon and nitrogen isotope ratios closely resemble those from the Spanish medieval site of Colegiata (Alexander et al., 2015). At Cacela, isotope data suggested a diet of both terrestrial and marine protein input. Males and females from this site exhibit a correlation between $\delta^{13}C$ and $\delta^{15}N$ values (Table 20), suggesting that marine protein input to diet was significant. Like the Convent of SF, the elevated $\delta^{13}C$ values at this site may be explained either by the consumption of C4 plants (i.e., millet), either directly or indirectly, or by marine protein.

Results of both the dietary modeling and the relationship between diet and BMD are discussed in the following chapter. This covers variation between sexes and sites, as well as attempts to variations by age. Essentially, Chapter 6 examines the results of the study based on the four proposed hypotheses in light of information gathered from historic and dietary modeling information.

Chapter 6 DISCUSSION

Incorporating multiple sources of information, this study has investigated the possible effect of dietary protein source on bone health and the etiology of osteoporosis (OP). The archaeological and historical records show that the intake of protein from both terrestrial and marine sources was common during the medieval period, and differed between regions, societal classes, and sex (Marques, 1971). A disease prevalent in both the past and present, OP is strongly influenced by diet. Though the effects of diet on bone health have been studied extensively in clinical settings, the etiology of OP can be explored further through the use of stable isotope ratios to interpret dietary protein source.

6.1 Variation Between Males and Females

The independent-samples T test results yielded an unexpected trend in the bone mineral density (BMD) variable (Table 12). The site of the Convent of SF showed no significant difference between BMD in males and females. This is unusual because physiological differences are expected between the sexes. However, this result is most likely influenced by the male sample consisting mainly of older diseased individuals (Table 15). This factor would skew the statistical results so that mean BMD appears similar in both males and females. Other aspects, such as activity patterns and peak bone mass differences may also have an effect on the lack of detectable difference in BMD. Additionally, unobservable diagenetic changes to the bone could have caused a spurious BMD values; however, it would then be expected that the female sample population would also exhibit inaccurate BMD values, thus still demonstrating the physiological separation of males and females.

The stable isotope variables of $\delta^{13}C$ and $\delta^{15}N$ illustrated no significant differences between the sexes, suggesting that diets between men and women at each site were similar (Table 18). This is not entirely unusual, and other studies into historic diet patterns have found similar results between the sexes (Nitsch, Humphrey, & Hedges, 2010). However, visual examination of the $\delta^{13}C$ and $\delta^{15}N$ values do show that males and females differ by approximately 0.4‰ for both variables (Table 6, Table 7, and Table 8). While this may not be a statistically significant difference it could be an indicator of differing dietary regimens between the sexes. Other studies have indicated sex-based dietary differences in medieval Iberian populations (Mundee, 2010). For this study, an increase in sample sizes would be necessary for determining whether there was a difference in diet between the sexes.

6.2 Variation by Site

One-way ANOVA analysis revealed that mean BMD differed by site. Bone mineral density varied significantly by sex and across all three sites indicating that distinct influences may be occurring at each site (Table 13). Whether or not these influences are mainly diet-related is difficult to pinpoint. Other factors that may have affected BMD are activity patterns, heritable influences, and diseases (Rosen, 2004).

The dietary variables $\delta^{13}C$ and $\delta^{15}N$ were also found to vary significantly between sites by sex (Table 19). Figure 11 illustrates the mean values of carbon and nitrogen for males and females at each site, demonstrating the significant differences between sites. Variations in stable isotope ratios may stem either from differences in diet or in baseline isotope values of food sources. While biological variables (e.g., sex) do not seem to play an influential role in diet structure within each site, it may be that cultural variables (e.g., status) have more of a hand in determining dietary norms between sites. This would explain the departure between the Convent of SF and the other two sites, since the Convent was a place where nobility was inhumed. However, it cannot be discounted that differing isoscapes could have contributed to inter-site dietary disparities. In addition to the distinct ecosystems and geologic diversity throughout Portugal, large trade systems that existed during the medieval era could contribute to variable $\delta^{13}C$ and $\delta^{15}N$ values (Waterman, 2012). Although information on Portugal's isoscapes is unavailable at this time, these considerations are a possibility that should be considered in the future.

6.3 Stable Isotope Results and Diet Modeling

Overall, the stable isotope results and One-way ANOVA analyses indicate that diets varied between sites, where sources of terrestrial and marine protein seem to have also varied (Table 19). Observation of the stable isotope mean values at each site suggests that different social classes, and possibly sexes, consumed somewhat distinct protein sources (Figure 23). Linear regression analyses, performed to explore the relationship of dietary protein source input at each site, suggested reliance on different sources of dietary protein.

At the SJ de Almedina site, the lack of correlation between $\delta^{13}C$ and $\delta^{15}N$ values suggests a diet mainly of terrestrial protein (Figure 24 and Figure 25). Because the cemetery was in an area likely to be utilized by the wealthy, as well as clergy members living within the medina, it serves to

reason then that their diets would have consisted of those protein sources associated with high status at the time (e.g., terrestrial protein sources such as beef) (Constable, 2012).

At the site of the Convent of SF the mean stable isotope ratios fall within the same range of $\delta^{13}C$ values as the other two sites, but $\delta^{15}N$ values are higher than comparative human values on the Iberian Peninsula and even surrounding Europe (Figure 30) (Alexander et al., 2015; Halffman & Velemínský, 2015; Lubell & Jackes, 1994). The linear regression analyses revealed a correlation between the two variables for both males and females, indicating a diet enriched with marine protein sources (Figure 26 and Figure 27). Because this cemetery was a burial site for noble and wealthy individuals, diets possibly followed religious prescriptions including the abstinence of terrestrial meat consumption and its subsequent replacement with fish. Why this would not also be observed at SJ de Almedina may be due to differences in the nitrogen content between marine and freshwater fish, but with both sites situated in riverine environments, differences are not likely to have been this substantial. Is it possible that the sample population from the Convent of SF consists mainly of monks and nuns who adhered much more strictly to religious edicts? Although monks and nuns would not have been considered high-status individuals, the convent was still in use at the time, and it is unknown where they would have been buried (Curate, personnel communication, March 10, 2015). This is a question for future study that could potentially explain the departure of the Convent SF's variables from the other two.

At Cacela-a-Velha the positive correlation between $\delta^{13}C$ and $\delta^{15}N$ values revealed a diet enriched with marine protein for males and females. Because $\delta^{13}C$ and $\delta^{15}N$ values from marine protein sources tend to be higher than those from terrestrial sources, individuals eating such fish will be enriched with both $\delta^{13}C$ and $\delta^{15}N$ (e.g., Ambrose et al., 1997; Lubell & Jackes, 1994) (Figure 28 and Figure 29). These findings fall within the context of historical records and make sense, considering the coastal location of the site. Meager grave goods and context indicate individuals interred here were not likely to have held high social positions, and with the area's background being one of a military stronghold coupled with a trade center, individuals were liable to subsist on readily available resources, such as marine protein.

Comparative data of human $\delta^{13}C$ and $\delta^{15}N$ values from the Iberian Peninsula, spanning the Neolithic through the medieval period, were also presented. All three sites in this study exhibit similar mean $\delta^{13}C$ values to that of the Spanish 13th to 16th century values, indicating the consumption of many similar resources. However, mean $\delta^{15}N$ values vary considerably. While

Neolithic societies were farming and subsisting mainly on terrestrial protein (e.g., meat, milk, plants), the earlier Mesolithic societies were exploiting marine protein sources (Lubell & Jackes, 1994). Even though the $\delta^{15}N$ values vary between the medieval sites, the majority fall within the expected range for the consumption of animal protein sources.

It is also possible that past land management practices, such as manuring, raise $\delta^{15}N$ values at the bottom of the food web; if separate sites practiced divergent land management practices this would affect and differentiate $\delta^{15}N$ values (Bogaard et al., 2007). In fact, this is another possibility for why the site of the convent of SF is so divergent from the other two sites in this study: differing land management practices by a community of monks and/or nuns would be consistent with its position in a popular city center. The need to produce viable crops from heavily utilized land would motivate supplementary manuring, ploughing, and burning of fields. Additional complications arise with the consumption of legumes (broad beans, peas) as they lower $\delta^{15}N$ values (Schoeninger, 2011). Consumption of these nitrogen fixing plants could possibly lower nitrogen isotope ratios at the other sites, circumstantially raising the nitrogen isotope ratios at the Convent SF; however, this still does not explain the large divergence of the Convent SF when compared to Mesolithic Portuguese individuals.

6.4 Variation by Disease Status

Normal and diseased (osteopenic and osteoporotic) individuals were compared across age and sex groups. Because osteoporosis is largely found in individuals of advanced age, changes in diet coupled with aging may have an effect on the prevalence of the disease. These subgroups were too small for statistical testing; but means for all subgroups within a site were generally within 1‰ for both $\delta^{13}C$ and $\delta^{15}N$. The small sample sizes preclude any more meaningful interpretation of the data.

6.5 Bone Mineral Density vs. $\delta^{13}C$ and $\delta^{15}N$

The results show no linear relationship between the variables except in two separate subgroups. The SJ de Almedina males and the Cacela females showed contradictory results; a positive correlation between nitrogen isotope ratios and BMD, and a negative correlation between carbon and nitrogen isotope ratios and BMD (respectively) (Table 17). The SJ de Almedina male sample population yielded no diseased individuals yet exhibited a significant positive relationship between BMD and nitrogen isotope values – as $\delta^{15}N$ increases, so does the mineral density of bone.

Of the men at SJ de Almedina, $\delta^{15}N$ values indicate a diet in line with values of inland dwellers from other parts of Europe (Halffman & Velemínský, 2015). Although it appears diets at SJ de Almedina consisted mainly of terrestrial protein sources (Figure 24 and Figure 25), these results suggest that a diet of terrestrial protein sources (e.g., beef, pork) has no detrimental effect on bone health.

The Cacela-a-Velha female sample population also yielded no diseased individuals yet exhibited a negative relationship between BMD and both nitrogen and carbon isotope ratios (Figure 21 and Figure 22). However, the negative correlation between these variables indicate a much different explanation from that of the males at S. J. de Almedina. Given that the correlation of carbon and nitrogen isotope ratios indicates the consumption of marine protein, this goes against the expectation that fish oil consumption should buffer the risk of bone loss (Fernandes, 2004). Where high $\delta^{13}C$ and $\delta^{15}N$ values are exhibited, BMD tends to be low. Additionally, bone may be responding as a buffer from the excessive amounts of dietary acid end products (Bushinsky, 2004; Sellmeyer et al., 2001). In this case, it appears that consuming marine protein sources has an unfavorable influence on BMD.

In summary, the first hypothesis considered as a part of this study suggested that a high ratio of animal to vegetable protein intake will increase bone loss and risk of fracture, which would be evident in a negative relationship between BMD and nitrogen isotope ratios. The Cacela female sample exhibits this correlation, providing some support for this hypothesis; however, evidence for the consumption of marine protein among this subgroup complicates this interpretation somewhat.

The second hypothesis suggested that a high ratio of animal to vegetable protein intake is associated with an increase in BMD, which would be evident in a positive linear association between BMD values and nitrogen isotope ratios. The results from the SJ de Almedina male sample exhibit this positive association, providing some support for this hypothesis.

The third hypothesis proposed that the consumption of seafood is associated with an increase in BMD, which would be evident in a positive linear association between BMD and for both nitrogen and carbon stable isotope ratios. The results from the Cacela female sample contradict this hypothesis, as they exhibit a negative association between BMD and both nitrogen and carbon isotope ratios. In this case, consumption of marine proteins do not appear to have had a positive impact on BMD.

Finally, the fourth hypothesis suggested that protein source may not have a significant effect on BMD, which would be evident in an outcome that exhibited no linear relationship between BMD

and the stable isotope ratios. The results from the Convent of SF support this hypothesis, as do the results from the SJ de Almedina females and the Cacela males. The lack of statistically significant results for the majority of subgroups indicates that the relationship between dietary protein sources and BMD is not straightforward. Essentially, the results presented here correspond with the findings in the clinical literature.

The contradictory findings at Cacela and SJ de Almedina may suggest that males and females metabolize sources of protein differently. While males and females differ physiologically in many ways, could their metabolism of protein differ to such an extent that dietary protein source would have opposite influences on BMD? While this extreme conclusion is unlikely, additional factors may, in fact, lie with differences in male/female hormone levels more specifically, relationships between insulin-like growth factor (IGF-1) levels and changes over an individuals' reproductive lifespan (Rickard, 2012). Influenced by dietary protein, IGF-1 plays a key role in bone metabolism where higher levels encourage bone growth (Heaney & Layman, 2008). It is well known that females undergo physiological changes throughout their lifespan that males contend with only minimally or not at all (e.g., menopause vs. andropause), and it may be the case that certain protein sources could in fact affect BMD adversely in women and not men.

However, why wouldn't these differences be observed in clinical studies in which men and women are investigated side by side? The answer may lie in the methods used by clinical researchers and their inability to test long-term dietary patterns directly. Through the use of bone collagen analysis, bioarchaeologists can directly investigate individual cumulative dietary patterns (over 10-30 years) and the physiological turnover of protein. Nevertheless, sample size often benefits the clinical researcher and inhibits the bioarchaeologist, which may also be why not all three sites of this study exhibited similar findings between males and females. An additional factor may also lie in the well accepted practice of prenatal vitamins in modern populations. The use of vitamins could be confounding the findings between modern and historic populations and the effects of protein metabolism during reproductive life stages (Cross, Hillman, Allen, Krause, & Viera, 1995). Interestingly, no relationship between isotopic variables and BMD was observed at the Convent of SF; as the site containing the greatest number of diseased individuals, some correlation between BMD and the dietary variables would be expected. Nonetheless, it may be the case that differing lifestyles weigh more heavily on the variation of BMD than diet (accounting also for the effects of this study's relatively small sample size).

Chapter 7 CONCLUSION

Based on clinical studies, the relationship between dietary protein source and osteoporosis (OP) is unclear. Studies have shown that animal and plant proteins are metabolized differently. Whether or not different sources of protein have a positive or negative effect on bone mineral density (BMD) is an area of great scientific interest, considering the high incidence of OP and the economic and individual strain of the disease.

The examination of past populations can reveal new insight into the role of nutrition on skeletal health. Stable isotope analysis allows bioarchaeologists to directly investigate dietary trends in past populations, and it provides the ability to do so in congruence with disease. This study investigated the relationship between health and diet in medieval Portugal by combining bioarchaeological data on the occurrence of OP (and BMD) with information on individual diet derived from stable isotope ratios.

At each of the three sites analyzed people consumed an omnivorous diet that included significant amounts of animal protein. Most of the plants consumed were C3 crops such as wheat, barley, and vegetables. Isotopic analyses also suggests a contribution of C4 plants, most likely millet. Marine fish was consumed by the individuals at the Convent de São Francisco and Cacela-a-Velha, and its contribution to diet at the site of the Convent of São Francisco suggests an unknown subgroup made up of individuals consuming a protein source high in ^{15}N. Though there were no statistically significant differences in diet based on sex, a small difference between males and females in nitrogen isotope ratios at each site may challenge these findings – larger samples sizes are certainly warranted. Further study of these aspects of sex and gender in past populations could contribute to a better understanding of disease processes.

Most of the regression analyses indicate no statistically significant relationship between BMD and either $\delta^{13}C$ or $\delta^{15}N$. However, for two of the subsamples a significant relationship between $\delta^{15}N$ and BMD was exhibited. The males at the site of São João de Almedina exhibit a statistically significant relationship between $\delta^{15}N$ values and BMD values at the $p = 0.001$ level, a positive correlation indicating that as $\delta^{15}N$ increases so does BMD. Interestingly enough, females at Cacela-a-Velha exhibited a negative correlation ($p = 0.032$) between BMD and $\delta^{15}N$ indicating the opposite relationship: as $\delta^{15}N$ increases, the density of bone decreases. However, because the majority of the findings indicate no relationship between dietary protein source, I propose that protein source has

no effect on the maintenance of BMD. Although dietary protein is critical to bone maintenance, dietary protein source may not be as critical.

There are limitations inherent to the study of multifactorial diseases such as osteoporosis. Other lifestyle and physiological factors such as activity, parity, and body mass (among others) have been shown to affect BMD – the major problem that this study shares with other epidemiological investigations is that it is merely a report of associations between individual characteristics. However, these results, along with the lack of a clear clinical relationship between BMD and protein source, suggest a complicated relationship between dietary protein source and the occurrence of osteoporosis. While samples sizes are small, the data indicate that future analysis is warranted, particularly considering the high incidence of osteoporosis and the economic and individual strain of the disease.

Future studies should incorporate larger sample sizes with more individuals from each sex, age, and social status group. Access to floral and faunal remains from the contexts studied would also be beneficial and may answer questions regarding anthropogenic influences on the environment (e.g., manuring practices) and provide a more comprehensive diet model for consumers. With these types of expanded information further insight into protein source may be obtained and reveal additional insight into the relationship between subgroups, their diets, and individual BMD.

Additional studies into differences in male and female physiology may also be important to the study of OP and diet. Larger samples of males and females that represent additional life stages may provide insight into the relationship between diet and hormones that affect bone maintenance. Because life stage hormone changes differ between males and females, consumption of similar protein sources may have differing influences upon each sex. This perspective of analysis may provide a deeper understanding of the etiology of OP.

In conclusion, the benefits of studying diet and its influences on OP from a bioarchaeological perspective are significant. Osteoporosis is a multifactorial disease influenced by many factors, and bioarchaeology provides an opportunity for investigating those influences in ways that cannot be achieved through clinical analysis. This study demonstrates the potential for future studies on the relationship of diet and osteoporosis, a disease that has affected people for thousands of years.

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